ASSISTED REPRODUCTION TECHNOLOGIES TO IMPROVE DAIRY CATTLE REPRODUCTION

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Victor Antonio Absalon-Medina

January 2014

ASSISTED REPRODUCTION TECHNOLOGIES TO IMPROVE DAIRY CATTLE REPRODUCTION

Victor Antonio Absalon-Medina, Ph. D.

Cornell University 2014

Over the past decades, dairy cattle reproduction has presented to farmers with several challenges as a consequence of genetic selection for improved milk production traits. These challenges include suboptimal postovulatory responses for timed artificial insemination synchronization protocols. Another example is the metabolic adjustments the preimplantation embryo may undergo in a high producing cow resulting in a high likelihood of early embryo loss. Nevertheless, this is an opportunity to study alternative options to improve pregnancy rates. Assisted Reproduction Technologies (ART) have the potential to solve several issues the modern dairy cow is facing. The progress in the practice of ART has been satisfactory over the last two decades and our understanding about gametes and embryo biology has substantially improved. Embryo transfers using in vitro produced embryos (IVP) might certainly have an advantage over conventional breeding methods since ovulation, fertilization and early embryonic stages would be bypassed thereby enhancing the likelihood of embryo implantation and hence improved conception rates. However, there are several challenges to producing good quality embryos in vitro due to difficulties in emulating the natural oviduct microenvironments that the preimplantation embryo is experiencing several physiological changes *en route* to the uterus. The objectives of this work were to review current literature in regard to mammalian preimplantation embryo production in vitro with emphasis in bovine species and to study the effect of metabolic regulators (MR) on embryo development as well as using new methods to recover better quality sperm especially when using sex-sorted semen.

Chapter Two is an extensive review of the IVP process in mammalian species with emphasis on the bovine embryo. Metabolic processes during oocyte in vitro maturation, sperm interactions during fertilization and in vitro cultures of different preimplantation embryo stages are reviewed. Special attention was devoted to the metabolic switch from low to high glucose uptake and metabolism occurring at the morula stage. Chapter Three is a systematic study of the effects of conjugated linoleic acid (CLA) isomers on embryos produced in vitro. Inclusion of 100 μM CLA- *cis* 9, *trans* 11 during embryo culture 36 hrs before cryopreservation resulted in embryos with higher survival and better developmental rates post-thaw when compared to other groups. Chapter Four presents a study about the effect of phenazine ethosulfate (PES) and 2, 4-dinitrophenol (DNP) on embryo development at the morula stage to enhance glucose uptake and metabolism to improve embryo developmental rates. Combination of 0.3 μM PES and 10 μM DNP resulted not only in higher embryo development and better quality but also embryos more resistant to cryopreservation procedures. Chapter Five describes a study of the effect of four colloidal-based sperm washes (Percoll, Old Bovipure, New Bovipure and Androcoll-B) on embryo development and quality. The International Embryo Transfer Society regulations strictly indicate that bovine embryos produced in vitro should be originated from sperm that has been recovered by silane-coated silica particles colloids when fractionation methods are used due to the debatable toxic effects of Percoll. Results from these experiments showed that embryos derived from sperm recovered by New Bovipure and Androcoll-B achieved higher blastocyst rates than Percoll and Old Bovipure groups. In addition, as a follow-up the effects of MR were evaluated on embryos originated from X-sorted semen. Interestingly, PES and DNP supplementation resulted in delayed development and poorer morphology in this embryos compared with untreated counterparts. Glucose

uptake threshold may be lower in female embryos and/or they may have a different substrate preference as compared to male embryos.

Our contribution to science may provide information for a better understanding of IVP and help shape the direction of future research. More importantly, it may provide the basis for production of better quality embryos originated from gender-selected semen with more chance of survival to term, thereby improving conception rates in dairy cattle.

BIOGRAPHICAL SKETCH

Victor Antonio Absalón-Medina was born on April 20, 1981 in Santiago Tuxtla, Veracruz, México, where he spent his childhood. He started a veterinary degree in 2000 at the Universidad Veracruzana Facultad de Medicina Veterinaria y Zootecnia. While he was studying for this degree he had an opportunity for an internship at the University of Wisconsin-La Crosse in 2002. In 2004 he served as a student volunteer for one year working with ranchers in the Sotavento and the Papaloapan regions of Veracruz, México. In 2005 he did another internship, this time at McGill University Macdonald campus in Quebec, Canada. After graduating from veterinary school he was accepted into the MS program in the Department of Animal Science at Cornell University in August 2005 with emphasis in Tropical Livestock Nutrition and International Agriculture and Rural Development supervised by Professor R.W. Blake. After successfully defending his MS thesis, in January of 2008 Victor entered into a Ph. D., program in Animal Science again in Cornell University with emphasis on Assisted Reproduction Technologies under the supervision of Professor W. R. Butler.

To my Family,

ACKNOWLEDGMENTS

I would like to express my gratitude to my immediate family: my mother, Maria Antonia Medina-Urbina; my grandmother, Maria Susana Martinez-Figueroa; and to my siblings, Nieves Guadalupe, Gabriel Augusto and Erik Alberto. Thank you for all the moral values you taught me during childhood and thank you for shaping the way I am today. In addition, thanks to all my family that from time to time provided additional help and moral support.

I would like to thank the motor of my life—Vanessa. Thank you for all these years of patience and dedication. I can only say that you are, besides an excellent mother, a very good spouse. My 5 year-old boy Victor Jose—you still do not get it but thank you for all the times you patiently waited and for the times when your father was physically at home but mentally at work.

Sylvia and Francisco Arrillaga I cannot thank you enough for being such a good friends to us. I truly appreciate the fact that you have always been supportive and especial thanks for all the excellent Argentinean-style gatherings with up-beat humor. I would like to show my appreciation and regards to my friend Nicholas Cappadona who has been really kind to me and my family since we arrived into Cornell.

I would like to thank my advisor, Professor W. R. Butler for giving me the opportunity to develop my ideas on ART and for his invaluable guidance. I would like to express my gratitude to him since without his help I would not have been able to complete the program. Thanks to my special committee members for the support provided and for helping review my dissertation. Thanks, Dr. M. E. van Amburgh and Dr. S.S. Suárez. Special thanks to Dr. R. O. Gilbert for providing lab facilities to conduct the IVF experiments.

I would like to thank Dr. M. A. Coutinho da Silva and Dr. S. J. Bedford-Guaus for all the teaching and support. I really appreciate the time you have dedicated showing me critical laboratory procedures.

Thanks to all my friends and colleagues that throughout my program have spent time helping me with the different experiments: Ocilon, Cheong, Chris, Lucas, Igor, Giulia and Augusto—Thanks for all the good times!

Thanks to the T& R crew especially to Gladys Birdsall, Raymond Axtell and Shawn McMahon since without their help I would have not been able to carry on with experiments that are not listed in this dissertation.

I am really grateful for all the support I received from Cargill Wyalusing, PA. John Couture and Lisa Ker-House thank you for helping me with the ovary orders to get the oocytes for the in vitro experiments. Likewise, infinite thanks to Genex for providing excellent quality cryopreserved semen. Thanks Dr. Michael Kaproth for being interested in our work and for the approval of the semen donation.

I am grateful for the economic support I received from CONACyT-México and Cornell University—Department of Animal Science. My PhD program would not have been a reality had I not receive such important support.

Almost done, Susanne Pelton thank you for learning how to deal with me, I really value every single moment you spent teaching me laboratory techniques and the logistics behind a laboratory work. I would like to thank Bruce Berggren-Thomas for all the help he provided and for being such a good person with me.

Last but not least, I would like to thank to all my friends from the Department of Animal Science for all the good times we have spent during the past years.

TABLE OF CONTENTS

CHAPTER ONE: INTRODUCTION

Livestock species are a very important source of food around the world. Over the last several decades, dairy cattle reproductive performance has gradually declined as genetic selection for milk traits has improved milk production (Butler, 2003). The modern dairy cow faces several challenges throughout her lifetime. From the moment of conception to preparturition, the presumptive embryo/fetus is constantly adapting to the uterine environment that is inherently related to the dam's metabolic status, especially toward the end of gestation (Bell, 1995). Moreover, a critical physiological stage for a cow is the transition period occurring three weeks pre- and three weeks post partum. Two important events occur at this stage: fetal overcrowding of the rumen in addition to the homeorhetic changes to accommodate the rather stressful lactation nutrient requirements postpartum. Suboptimal management during this physiological stage may result in retained placentas, subsequent poor reproductive performance and it seems that offspring performance could be also affected by this stressful situation.

An optimal maternal environment would provide the right nutrients/factors that will be reflected in the offspring's future performance under appropriate management conditions from birth throughout her entire lifetime (Absalón-Medina et al., 2009). Poor synchronization responses for timed artificial insemination, early pregnancy losses due to milk production stress, and suboptimal management conditions may constitute a large economic challenge for dairy producers (Sartori et al., 2010). For that reason, assisted reproduction technologies (ART) such as in vitro embryo production would provide the opportunity to farmers to bypass the aforementioned two critical steps thereby improving conception rates (Vasconcelos et al., 2006; Demetrio et al., 2007). Furthermore, ART have the potential to improve not only pregnancy rates, but also may provide producers opportunities to intelligently allocate

types of genetics and gender specificity in order to improve their current management programs for a likely increased demand of livestock products.

In vitro embryo production has evolved satisfactorily throughout the past three decades (reviewed in Chapter Two). Studies on murine and ruminant species have provided substantial knowledge about the different microenvironments the preimplantation embryo should experience for optimal development and for successful pregnancies. In addition, striking findings on the biology of oocyte maturation have unveiled critical factors, and the requirement for bidirectional communication with cumulus cells, to guarantee an optimal meiotic progress during this time in development (Gilchrist, 2011). In Chapter Two, we extensively review in vitro embryo production covering in vitro maturation, fertilization and the culture of preimplantation embryos throughout their different stages. Although there have been improvements in the protocols for production of embryos in vitro, there are still challenges that embryologists and clinicians face, such as the tradeoff between using defined media and media supplemented with serum; both approaches may result in negative outcomes but for different reasons. For example, defined media lack embryotrophic factors that are normally present in serum and serum-supplemented media may contain high levels of triglycerides and fatty acids that are detrimental to embryos. However, there are polyunsaturated fatty acids that reportedly become incorporated into cell membranes and provide membrane protection especially for embryos undergoing cryopreservation procedures (Pereira et al., 2008). Conjugated linoleic fatty acids (CLA) have been shown to act as signaling factors by regulation of fatty acid synthesis. A systematic study of the effects of CLA isomers during in vitro embryo production and cryopreservation procedures is described in Chapter Three.

Another subject of interest during preimplantation embryo culture is the regulation of glucose metabolism at the morula stage (Thompson et al., 2000; De La

Torre-Sanchez et al., 2006). Soon after fertilization, in vivo embryo development depends mostly on tricarboxylic acids such as pyruvate and lactate as sources of energy before reaching the morula stage. Coincident with the entrance to the hypoxic environment of the uterus, hexokinase activity is unregulated in order to allow more glucose uptake by the embryo for metabolism mainly via glycolysis and the pentose phosphate pathways. However, in vitro, this metabolic switch is not always activated in a pool of embryos and subsequently their development becomes arrested due to the inability to enhance glucose consumption and to meet the rapidly increasing energy requirements. Nonetheless, there are metabolic regulators that act as enhancers of glucose metabolism via the aforementioned pathways, thereby rescuing the pool of embryos that would otherwise fail. In Chapter Four we present our work on metabolic regulators provided at morula stage with the novel contribution of PES and DNP in combination to target simultaneously the two different metabolic pathways mentioned previously.

An important area during in vitro embryo production is the handling and processing of the semen. According to the International Embryo Transfer Society (IETS), semen has to be processed with colloidal solutions free of polyvinylpyrrolidone (PVP) since sperm toxicity has been reported when PVP is part of the colloid. Alternative macromolecules such as silane-coated silica particles have been recommended and approved by the IETS. In addition, these macromolecules have been reported to allow recovery of better quality sperm by close matching to their iso-pynic point (Morrell and Rodriguez-Martinez, 2010). In an effort to replace our Percoll (containing PVP) sperm fractionation procedures, we tested two different colloidal solutions containing silane-coated silica particles to measure their effect on computerized sperm motility parameters, fertilization rates and overall embryo development and quality (Chapter Five). Further, sex-sorted semen in vitro

fertilization (IVF) is a relatively new procedure that has the potential to provide several advantages to the livestock industry over conventional methods, especially for dairy cattle (Xu et al., 2009). For example, genetic improvements are expected to occur at a more rapid pace with enhanced efficiency of genetic selection of female replacements. Therefore, producers might reduce replacement heifer stocks and/or sell more heifers, which in turn could improve profitability. However, sex-sorted semen IVF to produce embryos of a desired gender is a new procedure, hitherto lacking a standard protocol. It is complicated because sires have different sperm-sorting and freezability threshold; thus, the sperm quality varies even more than when using conventional methods. As a result, IVF protocols that may work for one specific bull may not work for another (Xu et al., 2009). Another important area that may undoubtedly be inherent to sex-sorted semen IVF research is the development of media for embryos of a specific gender. It has been reported that there is a sex-based nutrient/substrate preference in embryos (Garcia-Herreros et al., 2012; Sturmey et al., 2009). Collectively, alternative colloidal-based sperm separators that would improve the efficiency of recovering good quality sperm, and thus better embryo development, along with addition of metabolic regulators to improve glucose consumption of embryos of a desired gender, were the objectives of our last experiments presented in Chapter Five.

REFERENCES

Absalón-Medina, V. A., R.W. Everett, M. E. Van Amburgh, and W. R. Butler. 2009. Imprinting effects of lactational performance from dam to calf during gestation. American Dairy Science Association (ADSA) Joint Meeting at Montreal, Canada. J. Anim. Sci. Vol. 87, E-Suppl. 2/J. Dairy Sci. Vol. 92, E-Suppl. 1. pp 274

Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J. Anim. Sci. 73:2804-2819.

Butler, W. R. 2003. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. Livest. Prod. Sci. 83: 211–218.

De La Torre-Sanchez, J. F., D. K. Gardner, K. Preis, J. Gibbons and G. E. Seidel Jr. 2006. Metabolic regulation of in vitro-produced bovine embryos. II. effects of phenazine ethosulfate, sodium azide and 2,4-dinitrophenol during post-compaction development on glucose metabolism and lipid accumulation. Reprod. Fertil. Dev. 18:597-607.

Demetrio DG, Santos RM, Demetrio CG, and Vasconcelos JL. 2007. Factors affecting conception rates following artificial insemination or embryo transfer in lactating Holstein cows. J Dairy Sci 90:5073-5082.

Garcia-Herreros, M., Aparicio, I. M., Rath, D., Fair, T., and Lonergan, P. 2012. Differential glycolytic and glycogenogenic transduction pathways in male and female bovine embryos produced *in vitro*. Reprod. Fertil. Dev. 24: 344-352.

Gilchrist, R. B. 2011. Recent insights into oocyte-follicle cell interactions provide opportunities for the development of new approaches to in vitro maturation. Reprod. Fertil. Dev. 23:23-31.

Morrell, J. M. and H. Rodriguez-Martinez. 2010. Practical applications of sperm selection techniques as a tool for improving reproductive efficiency. Vet. Med. Int. 2011:1-9.

Pereira, R. M., I. Carvalhais, J. Pimenta, M. C. Baptista, M. I. Vasques, A. E. Horta, I. C. Santos, M. R. Marques, A. Reis, M. S. Pereira and C. C. Marques. 2008. Biopsied and vitrified bovine embryos viability is improved by trans10, cis12 conjugated linoleic acid supplementation during in vitro embryo culture. Anim Reprod Sci 106:322-332.

Vasconcelos JL, Demetrio DG, Santos RM, Chiari JR, Rodrigues CA, and Sa Filho OG. 2006. Factors potentially affecting fertility of lactating dairy cow recipients. Theriogenology 65: 192-200.

Sartori, R., M. R. Bastos and M. C. Wiltbank. 2010. Factors affecting fertilisation and early embryo quality in single- and superovulated dairy cattle. Reprod Fertil Dev 22:151-158.

Sturmey, R. G., P. Bermejo-Alvarez, A. Gutierrez-Adan, D. Rizos, H. J. Leese and P. Lonergan. 2010. Amino acid metabolism of bovine blastocysts: A biomarker of sex and viability. Mol. Reprod. Dev. 77:285-296.

Thompson, J. G., C. McNaughton, B. Gasparrini, L. T. McGowan and H. R. Tervit. 2000. Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured in vitro. J. Reprod. Fertil. 118:47-55.

Xu, J., S. A. Chaubal and F. Du. 2009. Optimizing IVF with sexed sperm in cattle. Theriogenology. 71:39-47.

CHAPTER TWO: PRE-IMPLANTATION EMBRYO METABOLISM AND CULTURE SYSTEMS: CONSENSUS EXPERIENCE AMONG EUTHERIAN MAMMALS

This chapter provides an extensive literature review for the dissertation research projects and is formatted for submission as invited Review-Paper in the Journal of Assisted Reproduction and Genetics. The manuscript submission is due in September, 2013.

ABSTRACT

Despite advantages of in vitro embryo production in many species, widespread use of this technology is limited by generally lower developmental competence of in vitro derived embryos compared to in vivo counterparts. Regardless, *in vivo* or *in vitro* gametes and embryos face and must adjust to multiple microenvironments especially at preimplantation stages. Moreover, the ideal embryo has to be able to further adapt to environmental cues *in utero* to result in the birth of live and healthy offspring. Enormous strides have been made in understanding and meeting stage-specific requirements of preimplantation embryos, but interpretation of the data is more difficult due to the complexity of the wide array of culture systems and the remarkable plasticity of developing embryos that seem able to develop satisfactorily under a variety of conditions. Nevertheless, the objective remains meeting, as closely as possible, the preimplantation embryo requirements as it occurs in vivo. In general, oocytes and embryos develop more satisfactorily when cultured in groups. However, optimization of individual culture of oocytes and embryos is an important goal and area of intensive current research that will allow avoidance of ovarian superstimulation, with physiological and psychological advantages for patients. This review includes stage specific embryo requirements, culture systems including in vitro maturation, in vitro fertilization or intracytoplasmic sperm injection, and in vitro embryo culture, with a view to optimizing embryo culture in general, and culture of single embryos in particular.

INTRODUCTION

Genetic selection is constant and the main factor responsible for altering the genome of most species including humans. In domestic species, iatrogenic selection for desired traits has inadvertently resulted in an increased homozygosity stemming from the loss of variation in the chromosomal region flanking the selected allele and it is defined as selective sweeps (Ramey et al., 2013). As an example, dairy cattle reproduction decline has been related to genetic selection for improved milk production traits (Butler, 2003). One solution to overcome this issue is by using novel techniques offered in the field of assisted reproduction technologies.

The ultimate goal of assisted reproductive technologies (ART) is the birth of healthy offspring. Despite rapid progress in the field of ART for both human and veterinary medicine, several challenges remain. One example is that defined media still do not reflect the natural microenvironments of the female reproductive tract where gametes interact and embryos develop. Therefore, further optimization of culture conditions would provide a better environment especially for single oocytes, thereby circumventing problems associated with ovarian super stimulation and transfer of multiple embryos.

Defining optimal factors and substrates for the entire in vitro embryo production process has been an area of intensive research leading to understanding those most critical for early preimplantation embryonic development. However, the dynamic and complex nature of in vivo conditions makes it difficult to emulate such microenvironments in vitro with defined media. Complicating our assessment of culture methods is the plasticity of gametes and preimplantation embryos, with

acceptable development reported for a wide variety of culture conditions. Nonetheless, there are opportunities to define better culture systems that more closely match the physical and chemical interactions between gametes and embryos with the diverse microenvironments presented by the female reproductive tract. Improvements in culture systems should be focused on embryo metabolism at every stage of in vitro embryo production (IVP). In vivo, the embryo is exposed to a dynamic environment as its metabolic needs evolve from early to later preimplantation stages. Consumption of sodium pyruvate and other carboxylic acids is relatively greater at precompaction stages, coincident with the relatively more oxygenated environment of the oviduct. Once in the uterus, usually at compacted morula or early blastocyst stages, the oxygen tension is decreased and glucose metabolism increases to accommodate higher energy demands. This switch from low to high glucose consumption occurs in most species (Thompson et al., 2000; Gardner et al., 2013) and has become an important area for research in vitro. More optimal IVP systems would result in fewer "developmental block" issues and facilitate production of more high quality embryos for successful fresh-transfers or cryopreservation.

Here we review current understanding of optimal conditions for oocyte maturation (IVM), in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) and with more emphasis on in vitro embryo culture, pointing out existing obstacles and promising avenues of research for improvement. Our intention is to provide a comparative review of current knowledge of bovine and other non-human mammalian IVP models as a reference for potential application to human ART.

IN VITRO MATURATION

In vitro maturation in human ART is mostly used as a last resort, especially when patients do not respond well to superstimulation protocols; the resulting low maturation rates or overall poor developmental competence may potentially be inherent to the intrinsic quality of the oocyte and not due to the IVM procedure *per se*. Consequently, the poor IVM results have raised concerns and thus have limited the use of this procedure in human medicine (Banwell and Thompson, 2008; Smitz et al., 2011). In addition, the ICSI technique further limits the application of IVM for human oocytes since the first steps prior to and during fertilization are bypassed by the ICSI procedure. However, IVM may be suitable for patients with polycystic ovarian syndrome who are at higher risk of developing ovarian hyperstimulation syndrome when they undergo follicle superstimulation treatments (Banwell and Thompson, 2008).

There has been considerable progress in refining protocols for in vitro maturation of oocytes of livestock species with potential application to human IVM (Banwell and Thompson, 2008; Gilchrist, 2011). Controlling the delay of meiotic progress by means of adenylate cyclase activators, cyclic adenosine monophosphate (cAMP) analogues, or phosphodiesterase inhibitors is very critical during IVM. In addition, optimal conditions for cumulus cells surrounding the oocyte need to be considered as there is a complex, yet well orchestrated, bi-directional communication between these two cell types. Oocyte-secreted factors (OSF; Gilchrist, 2011) are involved in cumulus cell pathways that are directly related to the oocyte's welfare i.e. regulation of apoptosis, metabolism, proliferation, expansion and differentiation.

Recently, natriuretic peptide type C (NPPC) and its receptor (NPR2) were reported as essential for the maintenance of meiotic arrest in mouse oocytes (Zhang et al., 2011). The proposed mechanism in vitro is through production of cyclic guanosine monophosphate (cGMP) by exogenous NPPC binding to its receptor on cumulus cells. Consequently, cGMP is transferred, through gap junctions, to the oocyte where it plays a role in the inhibition of the phosphodiesterase 3A (PDE3A), thereby maintaining meiotic arrest by protecting cAMP levels from the degrading effects of active PDE3A. In vitro, exogenous estrogen seems to be required for the maintenance of NPR2 (Zhang et al., 2011). Collectively, cAMP and cGMP modulators along with OSF may result in improved protocols for in vitro maturation with potential application for ART.

During maturation, migration of mitochondria and lipid droplets to the center of the oocyte and location of cortical granules beneath the oolemma are good indicators of oocyte competence (Gordon, 2003). The novel maternal effect gene, peptidylarginine deiminase 6 (PADI6), which appears to be restricted in the mouse to the oocyte and early embryo, is required for the formation of cytoplasmic lattices and directly related to the formation of microtubules (Kan et al., 2010). The latter are responsible for the microorganelle redistribution during maturation and, thus, it is worthwhile to characterize this pathway to optimize IVM approaches. Since PADI6 is a Ca^{2+} sensitive enzyme, the role of Ca^{2+} oscillations is relevant in PADI6 function during maturation/fertilization (Malcuit et al., 2006).

Better non-invasive markers and improved ways to determine oocyte quality are needed, since morphological assessment is still the method of choice to select

"good" quality oocytes. As discussed by Goovaerts et al. (2010), new quality assessment protocols should meet specific criteria such as being practical, fast, and most importantly, reliable. Brilliant cresyl blue (BCB) has been reported as a good option for non-invasive assessment of oocyte and zygote quality (Koester et al., 2011). Although Goovaerts et al. (2010) detected no differences between control oocytes selected morphologically and BCB positive oocytes, a more recent report using BCB found that developmentally competent oocytes/zygotes exhibited lower zonar birefringence intensity parameters by polarized light microscopy (Koester et al., 2011).

The potential for providing an *assisted metabolism* to oocytes that have been exposed to a sub-optimal in vivo environment is a promising area for research. An altered follicular environment may result in changes in glucose metabolism (Sutton-McDowall et al., 2010) that profoundly affect viability of cumulus oocyte complexes during maturation. In cases of hyperglycemia, enzymes related to glycolysis such as hexokinase may become saturated and glucose may be repartitioned to other pathways such as the hexosamine biosynthesis (HBP [fuel sensing]) or Polyol pathways. Consequently, imbalances to the redox system may occur as well as undesirable O-linked glycosylation of serines and threonines normally responsive to kinases for activating other necessary pathways in meiotic progression (Sutton-McDowall et al., 2010). Nitration of these same amino acids might also be more common in an ammoniagenic environment (Lei et al., 2007) resulting from diets (e.g. ruminants) or media high in amino acids, thus, oocyte and embryonic developmental competence would be compromised.

Cumulus-oocyte complexes cultured in media supplemented with serum usually perform better than those cultured in serum-free media supplemented with synthetic macromolecules. However, in some situations, serum from patients (e.g. patients with recurrent pregnancy loss or contaminated commercial batches) may contain undesirable components that could potentially be detrimental for IVM. Evidence of rescuing factors, such as paraoxonase 1 (PON1 [in oocytes]) and preimplantation factor (PIF [in embryos]), suggests that oocytes may be improved by an optimal redox environment and thus increase the chances of fertilization or subsequent embryo development (Browne et al., 2008; Stamatkin et al., 2011a). Further research on these various factors is needed to better characterize under which conditions they might be beneficial as supplements for IVM. In addition, metabolic pathways in vitro are regulated by substrate availability in media and gas phase conditions (Banwell and Thompson, 2008; Bermejo-Alvarez et al., 2010). Exploring the latter options may result in optimization of IVM for a specific case when standard IVM conditions do not work due to a metabolic enzyme alteration.

IN VITRO FERTILIZATION/INTRACYTOPLASMIC SPERM INJECTION

Under natural conditions sperm travel through the utero-tubal junction to the isthmic region of the oviduct which acts as a functional sperm reservoir. Binder of sperm proteins, type 1, 3 and 5, (BSP; previously known as bovine seminal plasma proteins) have an active role in the binding of the sperm to the tubal epithelium and the release thereof during ovulation in order to target the matured egg (Gwathmey et al., 2006; Hung and Suarez, 2012). In addition, BSP proteins (BSP1 in particular) play

a role in sperm membrane configuration including removal of cholesterol and phospholipids during capacitation (Gordon, 2003; Gwathmey et al., 2003). In the isthmus, sperm bound to the luminal epithelium enjoy prolonged viability before undergoing capacitation and hyperactivation prior to fertilization (Gordon, 2003; Suarez, 2008). Although unknown, capacitation and hyperactivation have been thought of occurring simultaneously in vivo, however, different pathways for these processes have been demonstrated in vitro (Marquez and Suarez, 2004). In order to become capacitated (acrosome responsive), sperm have to undergo protein tyrosine phosphorylation via a cAMP/PKA signaling pathway. Hyperactivation is mediated by a Ca^{2+} signaling pathway.

Early fertilization events have been widely characterized in several species. Once the sperm is in the cytoplasm, the MII oocyte releases its cortical granule contents into the perivitelline space to avoid polyspermy. In addition, decondensation of the sperm head (replacement of protamines by maternal histone and non-histone proteins) takes place until the male pronucleus and pronuclear envelope are formed. Release of a spermatic phospholipase C_ζ (PLC_ζ) is needed for the hydrolysis of oocyte-cytoplasmic phosphatidylinositol-4, 5-bisphosphate (PIP_2) with formation of diacyl-glycerol phosphate (DAG) and inositol 1,4,5-tris-phosphate (IP_3). Subsequently these products stimulate resumption of meiosis by the arrested MII oocyte and extrusion of the second polar body followed by formation of female pronuclear envelope and pronucleus. This series of events depends on Ca^{2+} oscillations originated by sperm PLC_ζ that take place within a few hours after the spermatozoon enters the ooplasm. These oscillations are essential and result in downstream production of

molecules necessary for MII oocyte activation (reviewed in Kurokawa et al., 2004; Malcuit et al., 2006; Ducibella and Fissore, 2008). However, alternative mechanisms involved during oocyte activation seem to show a certain degree of redundancy. One example is the sperm-borne protein with conserved AA sequence throughout species, post-acrosomal WW domain binding protein (PAWP), that induces MII oocyte activation in a Ca^{2+} oscillation-related manner (Aarabi et al., 2010). Further, it has been shown that not only Ca^{2+} oscillations are needed for the progression of the cell cycle, but also zinc "sparks", a conserved mammalian mechanism, are required early after fertilization/activation (Kim et al., 2011). Collective integration of MII oocyte activation processes may need to be addressed.

Concerns about O_2 tension and its inherent relationship with the redox system have not only been the subject of investigation during IVM, but also at IVF. Studies by Lopes et al. (2010) and Bain et al. (2011) indicate a positive effect of higher O_2 tension during the first cellular cycle post IVF. In addition, overuse of antioxidants during bovine IVF impaired sperm quality, normal pronuclear formation, and embryo development to the blastocyst stage (Gonçalves et al., 2010; Marques et al., 2010). Thus, reactive oxygen species (ROS) may be critical for successful IVF, especially during the first cell cycle and therefore antioxidants and ROS scavengers should be used very judiciously (Lopes et al., 2010).

The technique of intracytoplasmic sperm injection (ICSI) is nearly unique as a means to achieve fertilization in the face of severe male infertility-related conditions (Merchant et al., 2011). Although ICSI is the most widely used technique for micro-assisted fertilization in humans, it remains inefficient for most other species due to

issues related to initiation of the normal cascade of events associated with activation of the arrested MII oocyte.

Over the last few years, several studies for improvements of ICSI in livestock have been conducted. The ICSI procedure has been used as an option to improve embryo rates in livestock species where IVF systems are currently inefficient (e.g. equine IVF). As reviewed by García Roselló et al. (2009), the future of ICSI, in laboratory and livestock species, will most likely deal with problems not associated with male infertility. Nevertheless, this will provide an excellent opportunity for refinement of protocols to optimize the technique. Malcuit et al. (2006), working with cattle, indicated that some of the signaling mechanisms that lead to the activation of the phosphoinositide pathway and generation of Ca^{2+} oscillations during natural fertilization were not replicated by ICSI. The problem appeared to stem from the inability to induce Ca^{2+} oscillations and that activation by an exogenous agent was required to initiate embryo development. Several methods for exogenous activation after ICSI have been reported such as applying calcium ionophores (e.g. ionomycin) in combination with 6-DMAP (Pereyra-Bonnet et al., 2008), ethanol (Abdalla et al., 2009), and roscovitine plus cytochalasin B (Devito et al., 2010). To date, global non-human blastocyst rate from ICSI remains at approximately 20 %.

Sperm selection based on physiological processes with potential practical applications have been reported (Morrell and Rodriguez-Martinez, 2010) where single layer centrifugation (SLC) and hyaluronan binding assays (HBA) are becoming the method of choice. Laser technology might be used to further improve ICSI, especially for livestock species as a model for potential human application. Recently, a real time

system was developed using laser technology and fluorescent imaging to measure individual sperm performance efficiently (Shi et al., 2011). This system offers an advantage for sperm selection since it allows measurement of mitochondrial membrane potential. Thus, studies of sperm energetics could be performed to characterize individual sperm not only from valuable high genetic merit animals and endangered species, but also from human patients being referred for IVF services. A possibility of improving ICSI results, at least in livestock species, may be to use this technology after the swim-up method to further optimize sperm selection from the pool of elite quality sperm (M.W. Berns, personal communication). Therefore, development of better approaches is needed to guarantee that the fertilizing spermatozoon expresses optimum performance i.e. capable of undergoing acrosome responsiveness, hyperactivation, and activation of the arrested MII oocyte. Along these lines, alternative options for oocyte activation after ICSI may need to be addressed such as the use of PLC_{ζ} or PAWP to trigger the necessary, more natural, Ca^{2+} oscillations or zinc sparks. Cloning research in livestock species may also be directly benefited by such studies.

EMBRYO CULTURE SYSTEMS

The existence of diverse embryo culture media and methods has made definition of the optimal components of embryo culture media very challenging. In vivo, the embryo is exposed to a dynamic environment as its metabolic needs evolve from early to later preimplantation stages. However, despite greater understanding of the biology of the embryo and its interaction with the oviduct and uterus, it has been

very difficult to emulate the different stage-regulated embryo metabolic pathways in vitro. Soon after fertilization the embryo benefits from a well oxygenated environment as in the oviduct and it shows substrate preference for tricarboxylic acids such as pyruvate and lactate. However, as the embryo develops to morula there is a metabolic switch that needs to be activated to supply an alternative source of energy in response to a more hypoxic environment in the uterus.

Emerging new technologies such as microfluidics, time-lapse cinematography and metabolomics may provide further advances in for producing good quality embryos in vitro before transfer to improve the likelihood of successful pregnancies and also to reduce the number of embryos transferred per cycle.

Preimplantation embryo metabolism and in vitro environment

Pioneering in vitro work by Yanagimachi and Chang (1964) on murine species led to important milestones in the creation of mammalian embryo culture media. From the 70's onwards, using murine models, findings of Bavister and coworkers resulted in major advances toward meeting embryo metabolic requirements (Bavister et al., 1983). During this time, Tervit and coworkers also made important progress on in vitro embryo culture by developing a synthetic oviduct fluid (SOF) medium based on biochemistry and physiology studies of the ovine oviduct (Tervit et al., 1972; Tervit et al., 1974). In addition, studies by Ménézo et al. (1976) provided insight on human in vitro embryo development. Critical work by Biggers and coworkers in the 80s and 90s, using mouse models and other mammalian species, created a sequential simplex optimization media (SOM), and later improvements (KSOM) allowed the 2-cell block

to be overcome at last (Lawitts and Biggers, 1991; Summers et al., 1995). Studies by Gardner and collaborators during this time led to important findings on embryo metabolism and creation of Gardner's media (Gardner et al., 1994). Furthermore, research work on livestock species resulted in critical improvements or development of new media such as Ménézo's B2 (Ménézo et al., 1984), CR1aa (Rosenkrans et al., 1993) and SOFaa (Holm et al., 1999). The most important milestones in development of modern embryo culture media are summarized in Table 1.

The extensive research activity carried on from ~1970-2000 was the basis of today's commercial media formulations for human and other mammalian species, but many of the advances in culture systems have been the result of empirical adjustments. As the embryo migrates from the oviduct to the uterus, a metabolic switch from low glucose to high glucose consumption occurs in most species (Thompson, 2000; Harvey, 2007; Feugang et al., 2009 [*vide* Figure 1]). Interestingly, equine embryos seem to show a different pattern in substrate preferences throughout preimplantation development, preferring hyperglycemic (>15 mM) and hypoxic conditions (5% CO_2, 5% O_2 [Hinrichs, 2010]). In any species, media formulation should account for this switch in metabolic pathways to most efficiently produce ATP depending on embryo-stage preference. Various chemical modulators and nutrients with established metabolic effects for optimizing embryo development are listed in Table 2. Further description of these metabolic effects is provided in the remainder of this section.

Oxidative metabolism is the main source for ATP especially at early (precompaction) embryonic stages, sodium pyruvate being the preferred tricarboxylic acid substrate. One of the earlier problems faced by researchers was the effect of

glucose in media at precompaction stages and the attendant undesirable early onset of glycolysis resulting in embryo growth retardation or embryo development block (reviewed by Thompson, 2000). However, supplementation of culture media with ethylenediaminetetraacetic acid (EDTA) overcame this issue with its inherent "Crabtree-effect" allowing embryos to continue further cell cycles (Thompson et al., 2000; Ménézo et al., 2013). After the problem of preventing early onset of glycolysis was solved by the addition of EDTA, attention turned to enhancement of glucose metabolism at postcompaction stages. Compounds with the opposite effect of EDTA not only enhance glucose consumption, but improve embryonic development and competence. Oxidative phosphorylation uncouplers such as 2, 4 dinitrophenol (DNP) partially inhibit oxidative phosphorylation while enhancing glycolysis (Thompson et al., 2000). Another metabolic regulator, phenazine ethosulfate (PES), is an electron acceptor for reduced nicotinamide adenine dinucleotide phosphate (NADPH) thereby enhancing the pentose phosphate pathway (De La Torre Sanchez et al., 2006). In addition, PES inhibits fatty acid synthesis (Sudano et al., 2011). Since DNP and PES both favor the metabolism of glucose, but via two different metabolic pathways, it would be of interest to evaluate the effects on the redox status of combining both chemicals under different glucose supplementation levels. Since these metabolic regulators may play a role in the fatty acid metabolism, the idea of combining their use with polyunsaturated fatty acid (PUFA) is also attractive.

Assisted metabolism may be necessary to aid in the regulation of glucose uptake in postcompaction in vitro produced embryos. This approach would permit improved efficiency of ATP production by competent embryos while also rescuing

embryos that would otherwise fail. Along these lines, Lopes et al. (2007) reported bovine embryo competence in relation to expression of glucose transporter 1 (GLUT-1) and glucose-6-phosphate dehydrogenase (G6PD) mRNAs. They demonstrated that metabolically active blastocysts use more oxygen and glucose than less competent embryos, indicating greater capability for glycolysis. Further, Leese et al. (2008) reported a functional metabolic "quietness" of viable preimplantation embryos, in contrast to impaired embryos which demonstrated increased energy consumption. Thus, there is a differential threshold between embryos with a certain degree of impairment showing higher (altered) metabolic rates and "quiet" embryos with an optimal degree of metabolic activity. Relevance of glucose transporters becomes critical, as embryo compaction occurs and glucose metabolism is enhanced, coincident with expression of insulin and IGF-I receptors. These receptors act in concert with glucose transporters, especially GLT-8, an insulin sensitive GLUT (reviewed by Purcell and Moley, 2009). According to a recent report, male and female bovine embryos have different requirements for glucose. Male embryos showed higher developmental rates compared to female embryos due, at least in part, to a higher metabolic rate of glucose (Garcia-Herreros et al., 2012; Table 2). This finding could significantly impact media formulation especially for the dairy or beef industry where the markets for female or male embryos are more popular, respectively.

Oxygen tension plays an important role in the expression of hypoxia inducible factors required for the up regulation of genes involved in the process of enhanced glucose metabolism and overall embryonic developmental competence postcompaction (Harvey, 2007). Today's embryo culture systems rely on a controlled

oxygen tension of 5-10% because there is extensive evidence for detrimental effects of higher oxygen tension in culture without supplementation of antioxidants or co-culture with somatic cells. Even the slightest increase in oxygen tension above the aforementioned range resulted in embryos with higher rates of apoptosis (among other developmental variables) than those cultured under controlled hypoxic conditions (5% $O_{2;}$ Arias et al., 2012).

Amino acid (AA) metabolism is gaining research interest in human and bovine species. Current sequential IVC systems provide the usual non-essential AAs during precompaction stages and a full array of essential and non-essential AAs at peri and postcompaction stages (reviewed by Gardner, 2008 and Feugang et al., 2009). Although providing an array of AA supports preimplantation embryo development, there has not been extensive research on the AA requirements at specific embryo stages. Besides the knowledge that alanine and glycine are the two major AAs required in higher concentrations for IVC, methionine is emerging as also being critical due to its interaction with folate (Kwong et al., 2010). The interaction of folate/methionine metabolism seems especially important because methionine is present in supraphysiological concentrations in contemporary culture media formulations, whereas, folate is not present in most IVC media formulations. The implications are that the embryo may deplete folate stores from the oocyte during advanced embryonic cellular cycles resulting in potential interruptions in metabolism. This adverse situation may be exacerbated with an excess of methionine available for intracellular trans-methylation and possible epigenetic alterations (Kwong et al., 2010). However, Bonilla et al. (2011) reported that DNA methylation of blastocyst

nuclei was unaffected by methionine concentration at several concentrations in vitro. In addition, the authors determined that methionine requirement during bovine preimplantation development is between 14-21 μmol/l and similar to the concentrations found in the bovine reproductive tract. The interaction of methionine and folate needs to be considered further.

Embryos of ruminants, as well as other mammals have requirements for both essential and non-essential AAs. Besides methionine, the essential AA lysine participates in metabolic pathways (e.g. donating carbon skeletons) and more importantly can be an epigenetic target site in histone tails to modulate chromatin structure (Monteiro et al., 2010). Another essential AA is histidine, which seems to be related to interferon tau expression (IFNτ; embryonic antiluteolytic cytokine required and secreted in ruminants for maternal recognition of pregnancy; Groebner et al., 2011). In addition to increased requirements for essential AAs as embryo development progresses, AA preference by gender has been reported recently. In vitro produced female embryos showed preference for arginine, glutamate and methionine whereas male embryos showed preference for phenylalanine, tyrosine and valine (Sturmey et al., 2010). For these reasons AA embryo requirements in vitro need to be established for improved development and conception rates per species.

According to metabolic and nutritional needs, the mammalian embryo shows a marked difference in requirements at early and late preimplantation stages. For this reason, sequential media have proved successful as extensively addressed by Gordon, 2003 and reviewed by Thompson, 2000; Gardner, 2008; Feugang et al., 2009; Hasler, 2010. Different sequential media formulations have been reported to achieve

acceptable rates of embryo development in vitro; however, there is usually limited information available on success rate following embryo transfer. Sequential culture strategies consider at least two phases covering pre-and-postcompaction stages, but, as discussed by Gruber et al. (2011), there remains much controversy regarding sequential vs. one-step culture with both similar and contradictory results.

The success of an IVP laboratory may stem not only from improvements of the IVC *per se*, but from the entire IVP system (Gardner, 2008). The latter includes: incubation conditions, gas phase, culture media, oil overlay, tissue culture ware/contact supplies, and embryo density and the volume of medium. In addition, embryologist or technician skills have to be considered as part of the system. One important area that deserves more attention is pH regulation in livestock embryo culture media. Emulation of physiological pH at the different preimplantation embryo stages may be critical for optimizing single and overall embryo culture systems. Therefore, the use of new pH zwitterionic buffering systems that would maintain a desired pH with minimal variations, during culture or handling, could result in more suitable environments for improved embryo development (Swain, 2010).

Defined media and supplements improving embryo production

Over the past 20 years a marked evolution in IVP systems has occurred from using surrogate xeno-oviducts and embryo co-culture conditions with somatic cells toward the optimum goal of producing embryos in defined serum-free media (Gordon, 2003; Hasler, 2010; Momozawa and Fukuda, 2011; Lazzari et al., 2011). The use of serum was the basis for the development of culture systems that did not require co-

culture conditions. Although supplementation of IVC media with serum or albumin is widely acknowledged to support improved embryo development relative to serum-free conditions, these supplements may also include detrimental factors that potentially impair embryo development. When using serum-containing media, a problem is encountered in culturing embryos destined to undergo cryopreservation procedures. Excessive accumulation of triglycerides may occur that results in detrimental effects on post-thaw performance. However, a recent study on mitochondrial ultrastructure assessed by transmission electron microscopy indicated no differences between embryos treated with serum vs. serum-free groups suggesting that serum may not be a major detrimental factor at least at the mitochondrial level before bovine embryo genome activation (EGA; Crocco et al., 2011). Nonetheless, for these and other reasons, such as minimizing the risk of disease transmission and evaluating the effect of specific factors during IVC on embryo performance, there is continuing demand for reliable and scientifically well defined cell and tissue culture methods (van der Valk et al., 2010). However, during the process of defining systems we must consider their adaptation to the physiological requirements of the embryo during culture (Thompson, 2000). Table 3 summarizes consensus results in the literature for blastocyst rates among the common livestock animals utilizing varied culture media formulations.

Optimal defined culture media for embryo production appears to require a combination of hormones, cytokines and antioxidants in order to replace serum or albumin with other macromolecules. Recently, more competent embryos have been reported when growth factors or cytokines are added either singly or in combination to the embryo culture media throughout the preimplantation stages. The major roles of

these factors are in regulation of apoptosis, cell differentiation and proliferation. Table 4 summarizes the effects of various growth factors and hormones for improving embryo development in defined media.

An important hormone for potential relevance in vitro is progesterone (P4) because of its requisite role in the maintenance of pregnancy. Results from trials studying the effects of P4 on preimplantation embryo development have been controversial. The hormone itself is lipophilic and in culture systems that usually rely on overlay of oil to avoid droplet evaporation, the P4 may be depleted from the media by the time embryos are transferred into the droplet. A study by Clemente et al. (2009) identified P4 receptors in preimplantation bovine embryos, but found no effect of in vitro P4 supplementation on elongated embryos post hatching. Considering earlier stages, embryos supplemented with a low dose of P4 (1 ng/ml) metabolized more glucose at postcompaction stages (Larson et al., 2011). Although P4 supplementation during culture did not improve embryo characteristics, these authors concluded that subtle changes in overall glucose partitioning and metabolism could have long term effects. In a more recent report, Ferguson et al. (2012) reported that the addition of P4 to the embryo culture stimulated the rate of embryo development and improved hatching rates. More research on effects of P4 in vitro seem warranted since the rise in circulating P4 levels that occurs after estrus and insemination is coincident with the embryo entering the uterus and the timing and switch from low to higher glycolytic rates.

A promising area for the improvement of single embryo culture systems is the utilization of embryonic lipid stores as an alternate source of energy especially for

species such as bovine, equine, and porcine where implantation occurs later than in human and murine species (Sturmey et al., 2009; Sutton-McDowall et al., 2012). In invertebrate species lipid droplets seem to regulate the function of proteins related to embryo genome activation (EGA) by maintaining them in an inactive state. Thus, lipid stores may function not only as a source of energy reserve, but also as a potential reservoir for signaling factors that play a role in cellular fate, in a spatiotemporal way, for mammalian early embryo development (Kim et al., 2012).

Oxidative stress and antioxidants

Most research on IVP has focused on the damaging effects of an oxidative environment and the inherent creation of reactive oxygen and nitrogen species (RONS) that may impair embryo development. The reduced glutathione (GSH) to oxidized glutathione (GSSG) ratio is the usual indicator of choice for measuring the redox status. Based on ratios of GSH/GSSG, the cytoplasm is a strong reducing environment whereas the endoplasmic reticulum (ER) is significantly less reducing (Rand and Grant, 2006). Consequently, excessive supplementation of reducing agents in media to offset oxidative damage has resulted in controversial outcomes as slight redox unbalances are detrimental for embryo development.

In eukaryotic cells there are two major systems responsible for maintaining a reduced state inside the cell. Glutathione and Thioredoxin (Trx) systems along with their respective reductases provide protection to proteins and enzymes during catalytic reactions in an interactive way (Rand and Grant, 2006; Koháryová and Kolárová, 2008) in order to maintain the reducing environment of the cell, to detoxify RONS

(Dunn et al., 2010), and to modulate various cellular activities such as gene expression, cell proliferation, survival and cell death (Sun and Regas, 2010). Disulfide bonds provide another mechanism of cellular protection and are essential for the folding and stability of proteins at the level of the ER. Under normal conditions, the most commonly used antioxidants such as β-mercaptoethanol (β-ME), GSH and taurine provide a buffer against oxidizing agents. However, an excess of antioxidants may compete for oxidizing equivalents in the ER and, thereby, promote an unfolded protein response due to an excessive accumulation of misfolded proteins (Rand and Grant, 2006). Consequently, an excess of reductants would disrupt not only multiple ER functions, but also signaling pathways that rely on low physiological concentrations of RONS as well as redox-sensitive transcription factors viz. *reducing stress* (Rand and Grant, 2006). Alternatively, an excess of RONS produced without sufficient antioxidant protection may lead to disequilibrium of the redox balance viz. *oxidative stress* characterized by RONS targeting and damaging DNA, RNA, protein and lipids (Koháryová and Kolárová, 2008).

Many attempts to minimize oxidative stress have been made during the past two decades. Culturing embryos under hypoxic conditions (i.e. 5-10% O_2) in combination with antioxidants partially compensated for the antioxidant contributions of co-culture cells. Studies using antioxidants under high and low oxygen tension conditions have resulted in controversial findings. Choe et al. (2010), using the swine model, studied the effect of the combination of GSH, β-ME and cysteine on embryo development. Treatment groups had a greater number of developing embryos than the control, however, higher concentrations of these antioxidants might have increased

embryo yield because high O_2 culture conditions (20% O_2 and 5% CO_2) were used. In contrast, Dovolu et al. (2011) working with guaiazulene (a component of various chamomile species with antioxidant properties) showed that under low oxygen tension (5% O_2) there was no positive effect on embryo rates when compared to control.

Melatonin has been reported to be a potent antioxidant. This hormone is a scavenger of free radicals and recent findings on melatonin either alone or in combination with taurine have been reported in buffalo and bovine species (Manjunatha et al., 2009; Takada et al., 2010). Overall, there were positive effects in the treatment groups compared to the controls for both studies, however, they used 20% O_2 and 5% CO_2 conditions. Thus, the use of antioxidants may yield greater benefits under high O_2 tension.

Recent attention has been given to phytochemicals containing antioxidant properties (Liu, 2004). For example, Jang et al. (2009) and Lee et al. (2011) tested the effects on bovine embryos of astaxanthin (carotenoids group) and 3, 4-dihydroxiflavone (phenolics group), respectively. Based on their culture conditions, they demonstrated that these phytochemicals had positive effects on cell proliferation, differentiation, and viability i.e. reduction of ROS, lipid peroxidation and apoptotic cell numbers.

Due to the increased interest in the redox systems and their interactions, more studies that not only measure the effectiveness of single factor supplementation, but also consider potential interactions of a cocktail of factors, antioxidant and hormones are needed, especially for single embryo culture. Lei et al. (2007) mentioned that expression of EGF receptor may participate in the regulation of glutathione

peroxidase-1 (GPX-1) along with GSH to maintain cellular homeostasis. Since both GPX-1 and Trxr are selenoenzymes, research on the interaction of both enzymes in light of the unfolded protein response status is of interest, especially when the GSH system belongs to the primary redox buffer that is competing with the Trx system for reducing equivalents. In addition, there seems to be an effect of preimplantation factor (PIF) on the regulation of Trx and GSH systems. This 15-AA polypeptide is a conserved sequence throughout mammalian species and it is produced by the viable embryo. Thus, integrating PIF on redox studies could provide more information on the regulation of the balance of such important systems.

The use of alternative macromolecules

Recent evidence has shown that embryos cultured in vitro remain inferior to those developed in vivo (Kuzmany et al., 2011). In addition, when embryos are cultured individually this inferior quality is more marked. However, culture of single embryos under defined conditions is highly desirable in human clinical situations, but presents challenges, e.g. single embryos (or COCs) in culture lack the benefits of embryotrophic factors from group culture and serum or albumin in the media. Variation from batch to batch of serum or albumin (i.e. contamination) is of concern and could potentially impair embryo development. Consequently, in an attempt to standardize the IVP production by making more defined culture media, many synthetic macromolecules have been used to replace animal-derived products. These include polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), ficoll and knockout serum replacement (KOSR [defined cocktail of factors]), but the results of using these

synthetic macromolecules have generally been disappointing. More promising results have been reported for recombinant human serum albumin (rHSA) in combination with hyaluronan (HA) that are the most abundant macromolecules in the female reproductive tract (Lane et al., 2003; Palasz et al., 2009). Table 5 summarizes different macromolecules and/or approaches to culture embryos.

Alternative new culture approaches

Under large-scale embryo production for livestock, group size in culture may not represent issues since the number of embryos per session typically allows groups of 10-25 embryos per droplet (usually 20-100 μL). By contrast, assisted reproduction clinics offer individualized services that represent a challenge in terms of group size culture conditions. Thus, reliable single (or small-group) COCs/embryo culture systems that can be replicated are necessary.

In a prospective study with human oocytes submitted to either IVF or ICSI, Ebner et al. (2010) tested a new type of culture dish and measured the effects of individual culture, individual culture with contact to neighbors, and group culture of zygotes (9-15). The results showed that group culture was best in terms of compaction and blastulation when compared to individual culture. Although there was only a trend for more births under group-culture treatment, the authors recommended group culture and also suggested a reduced volume of culture droplet or increased embryo density. Other studies investigating the effects of small-group or single embryo culture and continuous vs. sequential media have been encouraging. In mice, the effect of embryo density and microdrop volume on embryo development was tested (Vutyavanich et al.,

2011) and overall, no differences were found in terms of cleavage or blastocyst formation. However, embryos cultured singly had fewer ICM and TE cells than those cultured in groups. Nonetheless, groups as small as 2 embryos (in a microdrop from 0.5-2.0 μL) gave similar results to group culture in a 10 μL droplet. The effect of polyester mesh sections on the culture of COCs and embryos has been tested in an effort to reduce the labor inherent to the manual preparation for well of the well ("WOW") dishes. Results have indicated that oocyte maturation and embryo development may be carried out in polyester meshes (~170 μm opening) with similar developmental rates as those obtained by WOW or control group-culture (Somfai et al., 2010; Matoba et al., 2010).

A human IVF study investigated the effects of continuous uninterrupted single medium culture without replacement of fresh media vs. sequential media (Reed et al., 2009). No significant differences were detected between the two approaches in terms of embryo quality for d-3 transfers, however, for transfers at d-5 the continuous approach resulted in a greater number of suitable embryos than sequential media. In agreement, Paternot et al. (2010) demonstrated that although embryo quality was similar following uninterrupted culture vs. sequential media, the embryo utilization rate (embryos available for vitrification or fresh transfers over the total number of embryos) was higher for continuous culture. Interestingly a study using the bovine model reported that preimplantation embryonic development was better in a two-step sequential culture method than in the uninterrupted single media conditions as relative quantitative expression of gene markers related to embryo development showed higher values (Saadeldin et al., 2011). Furthermore, Kepkova et al. (2011) in a comparison of

two commercial culture media reported higher levels of relative mRNA expression in groups cultured in Ménézo B2 media indicating that the embryos were able to develop faster compared to those cultured in COOK media.

The emergence of microfluidic technology combining engineering and biology knowledge has led to a promising and innovative option for the in vitro culture of gametes and embryos in both static and dynamic ways. One of the obvious benefits of this technology would be the reduction of the "human factor" which would imply less manipulation of embryos and gametes, minimal fluctuations in the in vitro microenvironment and providing a more physiological nutrient delivery in the culture media as the embryo develops. Consequently, embryonic stress would be substantially reduced and the embryo viability could be enhanced (Feugang et al., 2009). Microfluidic approaches attempt to replicate more closely the early events of embryogenesis normally occurring during in vivo conditions (Krisher and Wheeler, 2010). The principle of this technology is basically a very small working volume of medium, in the nano-to-micro liter range. Two categories, static and dynamic, are recognized: in the dynamic system, the embryo remains stationary in a microwell in which media can be slowly changed. Krisher and Wheeler (2010) reviewed several prototypes of microfluidic systems, either static or dynamic, reporting overall positive results (equal to or better than control groups). However, in some cases it appeared there was decreased embryo development and these inconsistencies could not be explained. Current challenges to overcome are the material selection and the flow rate (Thompson, 2007; Feugang et al., 2009). Microfluidic technology is progressing rapidly as it seems that imagination is the only limiting factor when designing

microfluidic devices (Krisher and Wheeler, 2010). For example, Kim et al. (2009) developed a microfluidic in vitro culture system for mechanical stimulation of bovine embryos. Their system emulated the peristaltic constriction that normally occurs in vivo in the oviduct by designing microchannels with constricted areas. The results were positive and suggested that this constriction contributes to the early development of bovine embryos in ART systems.

Static microfluidic models using WOW systems have been used by several research groups and in most cases cleavage and blastocyst rates were not different compared to controls cultured in the usual groups. Taka et al. (2005) reported that WOW with a diameter of 1000 μm improved blastocyst rate in small groups (4-5/WOW) of swine embryos after ICSI (without exogenous activation) when compared to other groups (24.6 % vs. <13 %, respectively). Hoelker et al. (2010) concluded that a microenvironmental embryo density of 1:0.269 μL and a macroenvironmental embryo density of 1:30 μL were most successful for culturing bovine embryos in WOWs. The use of the WOW system to produce monozygotic twin bovine calves (Tagawa et al., 2008) using the blastomere separation technique resulted in higher pregnancy rates compared to the monozygotic twin blastocysts obtained by conventional bisection of in vivo derived blastocysts (78.9 vs. 40%). This may have a significant impact for the livestock industry and for research purposes as progeny would be derived from genetically valuable animals that could also be used for comparative studies by providing animals with an identical genetic background, thereby reducing the need for higher numbers of experimental units to reach statistical power.

Polydimethysilixane (PDMS) is widely used for manufacture of microfluidic devices due to its physical properties and low cost. Akagi et al. (2010) cultured bovine embryos using PDMS microwell plates and found no effect in terms of blastocyst development or the proportion of ICM and TE cells for embryos compared with those cultured in groups of 20 embryos without PDMS microwell plates. Han et al. (2010) and Ma et al. (2011) reported novel approaches using microfluidic devices capable of manipulating oocytes, which also enhanced embryo development. These positive results suggest that microfluidic devices simplify the overall IVP by reducing the human factor and also by tracking single embryo development.

Microfluidic technology allows the addition of a powerful tool such as time lapse cinematography (TLC) which could facilitate selection of the top quality embryos monitored in real time. TLC has lead to better understanding of fertilization and early preimplantation embryonic development that may have an impact later on pregnancy rates (Mio and Maeda, 2008; Niimura et al., 2010). Recently, Pribenszki et al. (2010) reported pregnancy and birth of a healthy infant in a single human IVF case using TLC in combination with WOW to select a single embryo for transfer. Although a single case is not sufficient to validate the usefulness of this approach, the authors encouraged application of TLC in combination with WOW. In bovine embryos, TLC in combination with polystyrene based-microwell culture resulted in pregnancy rates higher (51.7%) than control (21.9%) at day 60 post embryo transfer (Sugimura et al., 2010). This approach allowed the tracking and selection of the healthier bovine embryos for transfer. In addition, the risk of pregnancy loss was minimized. The authors did suggest that PDMS might absorb medium components such as

embryotrophic factors, making the novel polystyrene-WOW embryo culture system preferable.

The development of the “IVP lab-on-a-chip” would have a significant impact especially for human IVF since it would allow integrating oocyte positioning, sperm selection, fertilization medium replacement, embryo culture and monitoring of embryo development. Optimizing sinlge embryo culture conditions will play a pivotal role in the development of this new technology. In addition, the current challenge is integrating all the phases of the IVP into an “IVP-lab-on-a-chip” in vitro maturation and cumulus removal included on this device. Equal or higher quality standards than the current conventional methods and reliable results would be expected in order to be adopted by clinicians and embryologists.

Assessing embryo quality

In addition to the TLC, there are other non-invasive approaches for assessing the quality of embryos either for fresh transfer or cryopreservation. For example, assay of preimplantation factor (PIF) in media (Stamatkin et al., 2011b) may serve as a universal embryo viability marker since it is secreted by viable embryos of several species including mice, cattle and humans. This approach may represent a reliable tool to facilitate embryo selection for transfer into the recipient patient. Another non-invasive technique using the nanorespirometer to measure oxygen consumption as a marker of embryo viability has been reported (Lopes et al., 2010). Based on the same principle, the development of an embryoscope that combines time-lapse cinematography and an oxygen microsensor has been used to measure oxygen

consumption rates and to determine embryo developmental competence (Lopes et al., 2010).

Using amino acid profiling as another non-invasive approach, Sturmey et al. (2010) compared the developmental potential of bovine in vivo and in vitro derived embryos. They were able to detect differences between the two embryo sources, through increased AA media depletion by the in vitro group and also a sex-specific preference for amino acid depletion (Table 2). In humans, metabolomic profiling using near infrared spectroscopy is another effective non-invasive tool to predict embryo development (Seli et al., 2011). Non-invasive means of evaluating embryo quality and potential can be used to optimize decisions for embryo transfers (single vs. multiple) or cryopreservation procedures (by embryonic developmental stage) for maximum success (Guerif et al., 2009; Wang et al., 2010; Seli et al., 2011; Solé et al., 2011).

Although non-invasive approaches are improving, invasive ones have been extremely helpful in finding candidate genes to predict embryonic survival. Rekik et al. (2011) showed that it may be possible to establish transcriptomic landmarks specific for embryonic stage as critical markers for embryo quality. Bovine transcriptome analysis has revealed that part of the decreased reproductive rate in pure breed livestock species might be related to a high degree of inbreeding (Lazzari et al., 2011). Other transcriptomic differences have been reported for IVF blastocysts vs. degenerative embryos (Huang et al., 2010), in vivo vs. in vitro derived embryos (Kepkova et al., 2011), and blastocysts that did or did not result in pregnancy after transfer (Ghanem et al., 2011). In addition, within these analyses, the relative

expression of 18 genes was similar for in vivo and in vitro derived bovine embryos and whether resulting in delivery or not (Ghanem et al., 2011).

Interactions between the embryo and its maternal environment to consider for IVP

Initial gamete interaction, zygote development and embryo genome activation occur in vivo in the oviduct (Besenfelder et al., 2010). Embryo co-culture systems have been used to study maternal cross-talk during early preimplantation stages. Besenfelder et al. (2010) described the benefits for in vivo culture of in vitro derived bovine embryos using an oviduct culture model. The importance of the tubal-embryo interaction is demonstrated by surrogate xeno-oviducts supporting development of embryos that are more competent after transfer or more resistant to cryopreservation than their in vitro counterparts (Lazzari et al., 2010). Isolated mouse oviducts have been used for culturing bovine embryos. Although results were similar to in vitro culture, the oviduct demonstrated a remarkable immunotolerance for preimplantation embryos across species (Rizos et al., 2010). Co-culture of embryos with oviduct epithelial cells is a simpler model to study interactions of gametes and embryos than with the oviduct in vitro. Ulbrich et al. (2010) provided an extensive review on the bovine epithelial cell process for embryo co-culture systems. However, results should be interpreted with caution as the isolation of embryos and cells in this co-culture approach does not account for the more complex in vivo system.

A healthy endometrium is critical for pregnancy to occur especially in individuals undergoing repetitive ART. Fertility has been reported to be higher for cows with an endometrial thickness >10 mm after ovulation (Wiltbank et al., 2011).

Thus, an adequate thickness of the endometrium is recommended before embryo transfer. Neuromuscular electrical stimulation has been used in women to improve endometrial thickness and such treatment has resulted in a tendency for increased pregnancy rates compared to untreated controls (Bodombossou-Djobo et al., 2011). A thicker endometrium is associated with increased blood flow which may contain embryotrophic factors and substrates for a successful pregnancy and it is an important site for immunotolerance processes at the maternal-fetal interface. In a bovine study, a nonclassical MHC-I gene was reported to be responsive to cytokines that positively regulate pregnancy and suggests that these cytokines are not only involved in the preimplantation embryo survival per se, but also function as regulators at this interface (O'Gorman et al., 2010; Al Naib et al., 2011). Hill and Gilbert (2008) reported reduced overall cell number (particularly reduced trophectoderm cell number) in bovine embryos exposed to endometrial fluid recovered from a cow with uterine inflammation. The embryo-maternal environment interaction during pregnancy is complex and dynamic and future experiments toward single embryo culture in more defined conditions may benefit from studies integrating new knowledge from such interactions.

CONCLUSIONS AND FURTHER REMARKS

In the field of assisted reproductive technologies, in vitro derived embryos are still less developmentally competent than their in vivo counterparts. Several steps from in vitro maturation to fertilization and culture are not yet fully optimized in vitro. There is an increased awareness of the spatio-temporal requirements at several stages

of preimplantation embryo development and the different requirements for achieving optimal rates of development and quality. Although much can be learned from embryo co-culture systems that provide important insights, completely defined and optimized media is the goal. To obtain serum-free media for culturing embryos (especially single embryo culture) with minimum risk of disease transmission, there is a need to know the most critical nutrients, metabolic regulators, cytokines and hormones for each stage of embryo development. Animal models such as bovine IVP could be better exploited and results extrapolated to other species including the human. Important tools such as the non-invasive evaluation approaches for selection of elite quality embryos either for transfer or cryopreservation might be routinely used for minimizing the number of embryos transferred, assuring embryonic competence, and resulting in the birth of healthy offspring. Nevertheless, the use of invasive approaches such as transcriptome evaluation will continue to be a reliable tool to discover more relationships between embryonic markers and the maternal environment.

Conflict of interest

The authors declare no conflicts of interest

REFERENCES

Aarabi, M., Z. Qin, W. Xu, J. Mewburn and R. Oko. 2010. Sperm-borne protein, PAWP, initiates zygotic development in xenopus laevis by eliciting intracellular calcium release. Mol. Reprod. Dev. 77:249-256.

Abdalla, H., M. Shimoda, M. Hirabayashi and S. Hochi. 2009. A combined treatment of ionomycin with ethanol improves blastocyst development of bovine oocytes harvested from stored ovaries and microinjected with spermatozoa. Theriogenology. 72:453-460.

Akagi, S., M. Hosoe, K. Matsukawa, A. Ichikawa, T. Tanikawa and S. Takahashi. 2010. Culture of bovine embryos on a polydimethylsiloxane (PDMS) microwell plate. J. Reprod. Dev. 56:475-479.

Albuz, F. K., M. Sasseville, M. Lane, D. T. Armstrong, J. G. Thompson and R. B. Gilchrist. 2010. Simulated physiological oocyte maturation (SPOM): A novel in vitro maturation system that substantially improves embryo yield and pregnancy outcomes. Hum. Reprod. 25:2999-3011.

Al Naib, A., S. Mamo, G. M. O'Gorman, P. Lonergan, A. Swales and T. Fair. 2011. Regulation of non-classical major histocompatability complex class I mRNA expression in bovine embryos. J. Reprod. Immunol. 91:31-40.

Arias, M. E., R. Sanchez and R. Felmer. 2012. Evaluation of different culture systems with low oxygen tension on the development, quality and oxidative stress-related genes of bovine embryos produced in vitro. Zygote. 20: 209-217.

Ashkar, F. A., E. Semple, C. H. Schmidt, E. St John, P. M. Bartlewski and W. A. King. 2010. Thyroid hormone supplementation improves bovine embryo development in vitro. Hum. Reprod. 25:334-344.

Bain, N. T., P. Madan and D. H. Betts. 2011. The early embryo response to intracellular reactive oxygen species is developmentally regulated. Reprod. Fertil. Dev. 23:561-575.

Banwell, K. M. and J. G. Thompson. 2008. In vitro maturation of mammalian oocytes: Outcomes and consequences. Semin. Reprod. Med. 26:162-174.

Barnea ER, Sharma S. In: Infertility, Art & Endoscopy. Allahbadia GN, Merchant R, editor. Elsevier Pub; 2006. Prediction of Implantation in ART using molecular biology I; pp. 183–194.

Bavister, B. D., M. L. Leibfried and G. Lieberman. 1983. Development of preimplantation embryos of the golden hamster in a defined culture medium. Biology of Reproduction. 28:235-247.

Bermejo-Alvarez, P., P. Lonergan, D. Rizos and A. Gutierrez-Adan. 2010. Low oxygen tension during IVM improves bovine oocyte competence and enhances anaerobic glycolysis. Reprod. Biomed. Online. 20:341-349.

Besenfelder, U., V. Havlicek, A. Kuzmany and G. Brem. 2010. Endoscopic approaches to manage in vitro and in vivo embryo development: Use of the bovine oviduct. Theriogenology. 73:768-776.

Bevers, M. M. and F. Izadyar. 2002. Role of growth hormone and growth hormone receptor in oocyte maturation. Mol. Cell. Endocrinol. 197:173-178.

Bodombossou-Djobo, M. M., C. Zheng, S. Chen and D. Yang. 2011. Neuromuscular electrical stimulation and biofeedback therapy may improve endometrial growth for patients with thin endometrium during frozen-thawed embryo transfer: A preliminary report. Reprod. Biol. Endocrinol. 9:122-127.

Bonilla, L., D. Luchini, E. Devillard and P. J. Hansen. 2010. Methionine requirements for the preimplantation bovine embryo. J. Reprod. Dev. 56:527-532.

Browne, R. W., W. B. Shelly, M. S. Bloom, A. J. Ocque, J. R. Sandler, H. G. Huddleston and V. Y. Fujimoto. 2008. Distributions of high-density lipoprotein particle components in human follicular fluid and sera and their associations with embryo morphology parameters during IVF. Hum. Reprod. 23:1884-1894.

Butler, W. R. 2003. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. Livest. Prod. Sci. 83: 211–218.

Choe, C., Y. W. Shin, E. J. Kim, S. R. Cho, H. J. Kim, S. H. Choi, M. H. Han, J. Han, D. S. Son and D. Kang. 2010. Synergistic effects of glutathione and beta-mercaptoethanol treatment during in vitro maturation of porcine oocytes on early embryonic development in a culture system supplemented with L-cysteine. J. Reprod. Dev. 56:575-582.

Clemente, M., J. de La Fuente, T. Fair, A. Al Naib, A. Gutierrez-Adan, J. F. Roche, D. Rizos and P. Lonergan. 2009. Progesterone and conceptus elongation in cattle: A direct effect on the embryo or an indirect effect via the endometrium? Reproduction. 138:507-517.

Crocco, M., R. H. Alberio, L. Lauria and M. I. Mariano. 2011. Effect of serum on the mitochondrial active area on developmental days 1 to 4 in in vitro-produced bovine embryos. Zygote. 19: 297-306.

De La Torre-Sanchez, J. F., D. K. Gardner, K. Preis, J. Gibbons and G. E. Seidel Jr. 2006. Metabolic regulation of in vitro-produced bovine embryos. II. effects of phenazine ethosulfate, sodium azide and 2,4-dinitrophenol during post-compaction development on glucose metabolism and lipid accumulation. Reprod. Fertil. Dev. 18:597-607.

Deb, G. K., J. I. Jin, T. H. Kwon, B. H. Choi, J. I. Bang, S. R. Dey, I. R. Cho and I. K. Kong. 2011. Improved blastocyst development of single cow OPU-derived presumptive zygotes by group culture with agarose-embedded helper embryos. Reprod. Biol. Endocrinol. 9:121-131.

Devito, L. G., C. B. Fernandes, I. D. Blanco, P. M. Tsuribe and F. C. Landim-Alvarenga. 2010. Use of a piezo drill for intracytoplasmic sperm injection into cattle oocytes activated with ionomycin associated with roscovitine. Reprod. Domest. Anim. 45:654-658.

Dhali, A., Anchamparuthy, V. M., Butler, S. P., Mullarky, I. K., Pearson, R. E., Gwazdauskas, F. C. 2011. Development and quality of bovine embryos produced in vitro using growth factor supplemented serum-free system. Open Journal of Animal Sciences. 3: 97-105.

Dovolou, E., M. Clemente, G. Amiridis, I. Messinis, A. Kallitsaris, A. Gutierrez-Adan and D. Rizos. 2011. Effects of guaiazulene on in vitro bovine embryo production and on mRNA transcripts related to embryo quality. Reprod. Domest. Anim. 46:862-869.

Ducibella, T. and R. Fissore. 2008. The roles of Ca2+, downstream protein kinases, and oscillatory signaling in regulating fertilization and the activation of development. Dev. Biol. 315:257-279.

Dunn, L. L., A. M. Buckle, J. P. Cooke and M. K. Ng. 2010. The emerging role of the thioredoxin system in angiogenesis. Arterioscler. Thromb. Vasc. Biol. 30:2089-2098.

Ebner, T., O. Shebl, M. Moser, R. B. Mayer, W. Arzt and G. Tews. 2010. Group culture of human zygotes is superior to individual culture in terms of blastulation, implantation and life birth. Reprod. Biomed. Online. 21:762-768.

Felmer, R. N., M. E. Arias, G. A. Muñoz and J. H. Rio. 2011. Effect of different sequential and two-step culture systems on the development, quality, and RNA expression profile of bovine blastocysts produced in vitro. Mol. Reprod. Dev. 78:403-414.

Ferguson, C. E., D. J. Kesler and R. A. Godke. 2012. Progesterone enhances in vitro development of bovine embryos. Theriogenology. 77: 108-114.

Feugang, J. M., O. Camargo-Rodríguez and E. Memili. 2009. Culture systems for bovine embryos. Livestock Science. 121:141-149.

Fields, S. D., P. J. Hansen and A. D. Ealy. 2011. Fibroblast growth factor requirements for in vitro development of bovine embryos. Theriogenology. 75:1466-1475.

Garcia-Garcia, R. M., F. Ward, S. Fair, C. M. O'Meara, M. Wade, P. Duffy and P. Lonergan. 2007. Development and quality of sheep embryos cultured in commercial G1.3/G2.3 sequential media. Anim. Reprod. Sci. 98:233-240.

Garcia-Herreros, M., Aparicio, I. M., Rath, D., Fair, T., and Lonergan, P. 2012. Differential glycolytic and glycogenogenic transduction pathways in male and female bovine embryos produced *in vitro*. Reprod. Fertil. Dev. 24: 344-352.

Garcia-Rosello, E., E. Garcia-Mengual, P. Coy, J. Alfonso and M. A. Silvestre. 2009. Intracytoplasmic sperm injection in livestock species: An update. Reprod. Domest. Anim. 44:143-151.

Gardner, D. K., M. Lane, A. Spitzer and P. A. Batt. 1994. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: Amino acids, vitamins, and culturing embryos in groups stimulate development. Biol. Reprod. 50:390-400.

Gardner, D. K. 2008. Dissection of culture media for embryos: The most important and less important components and characteristics. Reprod. Fertil. Dev. 20:9-18.

Gardner, D. K. and P. L. Wale. 2013. Analysis of metabolism to select viable human embryos for transfer. Fertil. Steril. 99:1062-1072.

Ghanem, N., D. Salilew-Wondim, A. Gad, D. Tesfaye, C. Phatsara, E. Tholen, C. Looft, K. Schellander and M. Hoelker. 2011. Bovine blastocysts with developmental competence to term share similar expression of developmentally important genes although derived from different culture environments. Reproduction. 142:551-564.

Gilchrist, R. B. 2011. Recent insights into oocyte-follicle cell interactions provide opportunities for the development of new approaches to in vitro maturation. Reprod. Fertil. Dev. 23:23-31.

Goncalves, F. S., L. S. Barretto, R. P. Arruda, S. H. Perri and G. Z. Mingoti. 2010. Effect of antioxidants during bovine in vitro fertilization procedures on spermatozoa and embryo development. Reprod. Domest. Anim. 45:129-135.

Goovaerts, I. G., J. L. Leroy, E. P. Jorssen and P. E. Bols. 2010. Noninvasive bovine oocyte quality assessment: Possibilities of a single oocyte culture. Theriogenology. 74:1509-1520.

Goovaerts, I. G., J. L. Leroy, D. Rizos, P. Bermejo-Alvarez, A. Gutierrez-Adan, E. P. Jorssen and P. E. Bols. 2011. Single in vitro bovine embryo production: Coculture with autologous cumulus cells, developmental competence, embryo quality and gene expression profiles. Theriogenology. 76:1293-1303.

Gordon, I. ed. 2003. Laboratory Production of Cattle Embryos, 2nd Edition. Biotechnology in Agriculture Series, no. 27. pp 548.

Groebner, A. E., I. Rubio-Aliaga, K. Schulke, H. D. Reichenbach, H. Daniel, E. Wolf, H. H. Meyer and S. E. Ulbrich. 2011. Increase of essential amino acids in the bovine uterine lumen during preimplantation development. Reproduction. 141:685-695.

Gruber, I., and M. Klein. Embryo culture media for human IVF: Which possibilities exist? 2011. Journal of the Turkish-German Gynecological Association. 12: 110-117.

Guerif, F., M. Lemseffer, R. Bidault, O. Gasnier, M. H. Saussereau, V. Cadoret, C. Jamet and D. Royere. 2009. Single day 2 embryo versus blastocyst-stage transfer: A prospective study integrating fresh and frozen embryo transfers. Hum. Reprod. 24:1051-1058.

Gwathmey, T. M., G. G. Ignotz, J. L. Mueller, P. Manjunath and S. S. Suarez. 2006. Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the oviduct. Biol. Reprod. 75:501-507.

Gwathmey, T. M., G. G. Ignotz and S. S. Suarez. 2003. PDC-109 (BSP-A1/A2) promotes bull sperm binding to oviductal epithelium in vitro and may be involved in forming the oviductal sperm reservoir. Biology of Reproduction. 69:809-815.

Hambruch, N., J. D. Haeger, M. Dilly and C. Pfarrer. 2010. EGF stimulates proliferation in the bovine placental trophoblast cell line F3 via ras and MAPK. Placenta. 31:67-74.

Han, C., Q. Zhang, R. Ma, L. Xie, T. Qiu, L. Wang, K. Mitchelson, J. Wang, G. Huang, J. Qiao and J. Cheng. 2010. Integration of single oocyte trapping, in vitro fertilization and embryo culture in a microwell-structured microfluidic device. Lab Chip. 10: 2848-2854.

Harvey, A. J. 2007. The role of oxygen in ruminant preimplantation embryo development and metabolism. Anim. Reprod. Sci. 98:113-128.

Hashem, A., M. S. Hossein, J. Y. Woo, S. Kim, J. H. Kim, S. H. Lee, O. J. Koo, S. M. Park, E. G. Lee, S. K. Kang and B. C. Lee. 2006. Effect of potassium simplex optimization medium and NCSU23 supplemented with beta-mercaptoethanol and amino acids of in vitro fertilized porcine embryos. J. Reprod. Dev. 52:591-599.

Hasler, J. F. 2010. Synthetic media for culture, freezing and vitrification of bovine embryos. Reprod. Fertil. Dev. 22:119-125.

Hill, J. and R. Gilbert. 2008. Reduced quality of bovine embryos cultured in media conditioned by exposure to an inflamed endometrium. Aust. Vet. J. 86:312-316.

Hinrichs, K. 2010. In vitro production of equine embryos: State of the art. Reprod. Domest. Anim. 45 Suppl 2:3-8.

Hinrichs, K. 2012. Assisted reproduction techniques in the horse. Reprod. Fertil. Dev. 25:80-93.

Hoelker, M., F. Rings, Q. Lund, C. Phatsara, K. Schellander and D. Tesfaye. 2010. Effect of embryo density on in vitro developmental characteristics of bovine preimplantative embryos with respect to micro and macroenvironments. Reprod. Domest. Anim. 45:e138-e45.

Holm, P., P. J. Booth, M. H. Schmidt, T. Greve and H. Callesen. 1999. High bovine blastocyst development in a static in vitro production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology. 52:683-700.

Hoshi, H. 2003. In vitro production of bovine embryos and their application for embryo transfer. Theriogenology. 59:675-685.

Hu, J., D. Cheng, X. Gao, J. Bao, X. Ma and H. Wang. 2012. Vitamin C enhances the in vitro development of porcine pre-implantation embryos by reducing oxidative stress. Reproduction in Domestic Animals. 47:873-879.

Huang, W., B. S. Yandell and H. Khatib. 2010. Transcriptomic profiling of bovine IVF embryos revealed candidate genes and pathways involved in early embryonic development. BMC Genomics. 11: 23-33.

Hugentobler, S. A., J. M. Sreenan, P. G. Humpherson, H. J. Leese, M. G. Diskin and D. G. Morris. 2010. Effects of changes in the concentration of systemic progesterone on ions, amino acids and energy substrates in cattle oviduct and uterine fluid and blood. Reprod. Fertil. Dev. 22:684-694.

Hung, P. and S. S. Suarez. 2012. Alterations to the bull sperm surface proteins that bind sperm to oviductal epithelium. Biology of Reproduction. 87:88-98.

Jang, H. Y., S. J. Ji, Y. H. Kim, H. Y. Lee, J. S. Shin, H. T. Cheong, J. T. Kim, I. C. Park, H. S. Kong, C. K. Park and B. K. Yang. 2010. Antioxidative effects of astaxanthin against nitric oxide-induced oxidative stress on cell viability and gene expression in bovine oviduct epithelial cell and the developmental competence of bovine IVM/IVF embryos. Reprod. Domest. Anim. 45:967-974.

Kan, R., P. Yurttas, B. Kim, M. Jin, L. Wo, B. Lee, R. Gosden and S. A. Coonrod. 2011. Regulation of mouse oocyte microtubule and organelle dynamics by PADI6 and the cytoplasmic lattices. Dev. Biol. 350:311-322.

Kepkova, K. V., P. Vodicka, T. Toralova, M. Lopatarova, S. Cech, R. Dolezel, V. Havlicek, U. Besenfelder, A. Kuzmany, M. A. Sirard, J. Laurincik and J. Kanka. 2011. Transcriptomic analysis of in vivo and in vitro produced bovine embryos revealed a developmental change in cullin 1 expression during maternal-to-embryonic transition. Theriogenology. 75:1582-1595.

Kim, A. M., M. L. Bernhardt, B. Y. Kong, R. W. Ahn, S. Vogt, T. K. Woodruff and T. V. O'Halloran. 2011. Zinc sparks are triggered by fertilization and facilitate cell cycle resumption in mammalian eggs. ACS Chem. Biol. 6:716-723.

Kim, E. Y., J. B. Lee, H. Y. Park, C. J. Jeong, K. Z. Riu and S. P. Park. 2011. The use of embryonic stem cell derived bioactive material as a new protein supplement for the in vitro culture of bovine embryos. J. Reprod. Dev. 57:346-354.

Kim, J., B. Song, K. Lee, D. Kim, S. Kim, Y. Choo, K. Chang and D. Koo. 2012. Tauroursodeoxycholic acid enhances the pre-implantation embryo development by reducing apoptosis in pigs. Reproduction in Domestic Animals. 47:791-798.

Kim, K., S. Park and S. Roh. 2012. Lipid-rich blastomeres in the two-cell stage of porcine parthenotes show bias toward contributing to the embryonic part. Anim. Reprod. Sci. 130:91-98.

Kim, M. S., C. Y. Bae, G. Wee, Y. M. Han and J. K. Park. 2009. A microfluidic in vitro cultivation system for mechanical stimulation of bovine embryos. Electrophoresis. 30:3276-3282.

Koester, M., A. Mohammadi-Sangcheshmeh, M. Montag, F. Rings, T. Schimming, D. Tesfaye, K. Schellander and M. Hoelker. 2011. Evaluation of bovine zona pellucida characteristics in polarized light as a prognostic marker for embryonic developmental potential. Reproduction. 141:779-787.

Koharyova, M. and M. Kolarova. 2008. Oxidative stress and thioredoxin system. Gen. Physiol. Biophys. 27:71-84.

Kolle, S., M. Stojkovic, G. Boie, E. Wolf and F. Sinowatz. 2002. Growth hormone inhibits apoptosis in in vitro produced bovine embryos. Mol. Reprod. Dev. 61:180-186.

Kolle, S., M. Stojkovic, S. Reese, H. D. Reichenbach, E. Wolf and F. Sinowatz. 2004. Effects of growth hormone on the ultrastructure of bovine preimplantation embryos. Cell Tissue Res. 317:101-108.

Krisher, R. L. and M. B. Wheeler. 2010. Towards the use of microfluidics for individual embryo culture. Reprod. Fertil. Dev. 22:32-39.

Kurokawa, M., K. Sato and R. A. Fissore. 2004. Mammalian fertilization: From sperm factor to phospholipase czeta. Biol. Cell. 96:37-45.

Kuzmany, A., V. Havlicek, G. Brem, I. Walter and U. Besenfelder. 2011. Assessment of actin cytoskeleton and nuclei in bovine blastocysts developed under different culture conditions using a novel computer program. Reprod. Domest. Anim. 46:46-53.

Kuzmina, T. I., H. Alm, V. Denisenko, A. Tuchscherer, W. Kanitz and H. Torner. 2007. Effect of recombinant bovine somatotropin (rbST) on cytoplasmic maturation of bovine oocytes and their developmental competence in vitro. J. Reprod. Dev. 53:309-316.

Kwong, W. Y., S. J. Adamiak, A. Gwynn, R. Singh and K. D. Sinclair. 2010. Endogenous folates and single-carbon metabolism in the ovarian follicle, oocyte and pre-implantation embryo. Reproduction. 139:705-715.

Lagutina, I., G. Lazzari and C. Galli. 2006. Birth of cloned pigs from zona-free nuclear transfer blastocysts developed in vitro before transfer. Cloning Stem Cells. 8:283-293.

Lane, M., J. M. Maybach, K. Hooper, J. F. Hasler and D. K. Gardner. 2003. Cryo-survival and development of bovine blastocysts are enhanced by culture with recombinant albumin and hyaluronan. Mol. Reprod. Dev. 64:70-78.

Larson, J. E., R. L. Krisher and G. C. Lamb. 2011. Effects of supplemental progesterone on the development, metabolism and blastocyst cell number of bovine embryos produced in vitro. Reprod. Fertil. Dev. 23:311-318.

Lawitts, J. A. and J. D. Biggers. 1991. Overcoming the 2-cell block by modifying standard components in a mouse embryo culture medium. Biology of Reproduction. 45:245-251.

Lazzari, G., S. Colleoni, R. Duchi, A. Galli, F. D. Houghton and C. Galli. 2011. Embryonic genotype and inbreeding affect preimplantation development in cattle. Reproduction. 141:625-632.

Lazzari, G., S. Colleoni, I. Lagutina, G. Crotti, P. Turini, I. Tessaro, D. Brunetti, R. Duchi and C. Galli. 2010. Short-term and long-term effects of embryo culture in the surrogate sheep oviduct versus in vitro culture for different domestic species. Theriogenology. 73:748-757.

Lee, K. S., E. Y. Kim, K. Jeon, S. G. Cho, Y. J. Han, B. C. Yang, S. S. Lee, M. S. Ko, K. J. Riu, H. T. Lee and S. P. Park. 2011. 3,4-dihydroxyflavone acts as an antioxidant and antiapoptotic agent to support bovine embryo development in vitro. J. Reprod. Dev. 57:127-134.

Leese, H. J., C. G. Baumann, D. R. Brison, T. G. McEvoy and R. G. Sturmey. 2008. Metabolism of the viable mammalian embryo: Quietness revisited. Mol. Hum. Reprod. 14:667-672.

Lei, X. G., W. H. Cheng and J. P. McClung. 2007. Metabolic regulation and function of glutathione peroxidase-1. Annu. Rev. Nutr. 27:41-61.

Liu, R. H. 2004. Potential synergy of phytochemicals in cancer prevention: Mechanism of action. J. Nutr. 134:3479-3485.

Lopes, A. S., M. Lane and J. G. Thompson. 2010. Oxygen consumption and ROS production are increased at the time of fertilization and cell cleavage in bovine zygotes. Hum. Reprod. 25:2762-2773.

Lopes, A. S., C. Wrenzycki, N. B. Ramsing, D. Herrmann, H. Niemann, P. Lovendahl, T. Greve and H. Callesen. 2007. Respiration rates correlate with mRNA expression of G6PD and GLUT1 genes in individual bovine in vitro-produced blastocysts. Theriogenology. 68:223-236.

Loureiro, B., J. Block, M. G. Favoreto, S. Carambula, K. A. Pennington, A. D. Ealy and P. J. Hansen. 2011a. Consequences of conceptus exposure to colony-stimulating factor 2 on survival, elongation, interferon-tau secretion, and gene expression. Reproduction. 141:617-624.

Loureiro, B., L. J. Oliveira, M. G. Favoreto and P. J. Hansen. 2011b. Colony-stimulating factor 2 inhibits induction of apoptosis in the bovine preimplantation embryo. Am. J. Reprod. Immunol. 65:578-588.

Ma, R., L. Xie, C. Han, K. Su, T. Qiu, L. Wang, G. Huang, W. Xing, J. Qiao, J. Wang and J. Cheng. 2011. In vitro fertilization on a single-oocyte positioning system

integrated with motile sperm selection and early embryo development. Anal. Chem. 83:2964-2970.

Makarevich, A. V., E. Kubovicova, Z. Hegedusova, J. Pivko and F. Louda. 2012. Post-thaw culture in presence of insulin-like growth factor I improves the quality of cattle cryopreserved embryos. Zygote. 20: 97-102.

Malcuit, C., M. Maserati, Y. Takahashi, R. Page and R. A. and Fissore. 2006. Intracytoplasmic sperm injection in the bovine induces abnormal [Ca2+]i responses and oocyte activation. Reprod. Fertil. Dev. 18: 39-51.

Malcuit, C., M. Kurokawa and R. A. Fissore. 2006. Calcium oscillations and mammalian egg activation. J. Cell. Physiol. 206:565-573.

Manjunatha, B. M., M. Devaraj, P. S. Gupta, J. P. Ravindra and S. Nandi. 2009. Effect of taurine and melatonin in the culture medium on buffalo in vitro embryo development. Reprod. Domest. Anim. 44:12-16.

Marques, A., P. Santos, G. Antunes, A. Chaveiro and F. Moreira da Silva. 2010. Effect of alpha-tocopherol on bovine in vitro fertilization. Reprod. Domest. Anim. 45:81-85.

Marquez, B. and S. S. Suarez. 2004. Different signaling pathways in bovine sperm regulate capacitation and hyperactivation. Biol. Reprod. 70:1626-1633.

Matoba, S., T. Fair and P. Lonergan. 2010. Maturation, fertilisation and culture of bovine oocytes and embryos in an individually identifiable manner: A tool for studying oocyte developmental competence. Reprod. Fertil. Dev. 22:839-851.

Matsukawa, K., S. Akagi, N. Adachi, F. Sato, T. Hasegawa and S. Takahashi. 2007. In vitro development of equine oocytes from preserved ovaries after intracytoplasmic sperm injection. Reprod. Dev. 53:877-885.

Ménézo, Y. 1976. Milieu synthétique pour ia survie et la maturation des gamètes et pour la culture de l'œuf fécondé. CR Acad. Sci Paris, ser D. 282: 1967-1970.

Ménézo, Y., J. Testart and D. Perrone. 1984. Serum is not necessary in human in vitro fertilization, early embryo culture, and transfer. Fertil. Steril. 42: 750-755.

Ménézo, Y., I. Lichtblau and K. Elder. New insights into human pre-implantation metabolism in vivo and in vitro. 2013. JARG. 30: 293-303.

Merchant, R., G. Gandhi and G. N. Allahbadia. 2011. In vitro fertilization/intracytoplasmic sperm injection for male infertility. Indian. J. Urol. 27:121-132.

Mingoti, G. Z., V. S. Castro, S. C. Meo, L. S. Sa Barretto and J. M. Garcia. 2011. The effects of macromolecular and serum supplements and oxygen tension during bovine in vitro procedures on kinetics of oocyte maturation and embryo development. In Vitro Cell. Dev. Biol. Anim. 47:361-367.

Mio, Y. and K. Maeda. 2008. Time-lapse cinematography of dynamic changes occurring during in vitro development of human embryos. Am. J. Obstet. Gynecol. 199:660.-665.

Momozawa, K. and Y. Fukuda. 2011. Establishment of an advanced chemically defined medium for early embryos derived from in vitro matured and fertilized bovine oocytes. Reprod. Dev. 57: 681-689

Monteiro, F. M., C. S. Oliveira, L. Z. Oliveira, N. Z. Saraiva, M. E. Mercadante, F. L. Lopes, D. R. Arnold and J. M. Garcia. 2010. Chromatin modifying agents in the in vitro production of bovine embryos. Vet. Med. Int. 2011:694817.

Moreira, F., F. F. Paula-Lopes, P. J. Hansen, L. Badinga and W. W. Thatcher. 2002. Effects of growth hormone and insulin-like growth factor-I on development of in vitro derived bovine embryos. Theriogenology. 57:895-907.

Morrell, J. M. and H. Rodriguez-Martinez. 2010. Practical applications of sperm selection techniques as a tool for improving reproductive efficiency. Vet. Med. Int. 2011:1-9.

Neira, J. A., D. Tainturier, M. A. Pena and J. Martal. 2010. Effect of the association of IGF-I, IGF-II, bFGF, TGF-beta1, GM-CSF, and LIF on the development of bovine embryos produced in vitro. Theriogenology. 73:595-604.

Niimura, S., T. Ogata, A. Okimura, T. Sato, Y. Uchiyama, T. Seta, H. Nakagawa, K. Nakagawa and Y. Tamura. 2010. Time-lapse videomicrographic observations of blastocyst hatching in cattle. J. Reprod. Dev. 56:649-654.

O'Gorman, G. M., A. Al Naib, S. A. Ellis, S. Mamo, A. M. O'Doherty, P. Lonergan and T. Fair. 2010. Regulation of a bovine nonclassical major histocompatibility complex class I gene promoter. Biol. Reprod. 83:296-306.

Palasz, A. T., P. Beltran Brena, J. De la Fuente and A. Gutierrez-Adan. 2010. The effect of bovine embryo culture without proteins supplements until day 4 on transcription level of hyaluronan synthases, receptors and mtDNA content. Zygote. 18:121-129.

Paternot, G., S. Debrock, T. M. D'Hooghe and C. Spiessens. 2010. Early embryo development in a sequential versus single medium: A randomized study. Reprod. Biol. Endocrinol. 8: 83-89.

Pereyra-Bonnet, F., R. Fernandez-Martin, R. Olivera, J. Jarazo, G. Vichera, A. Gibbons and D. Salamone. 2008. A unique method to produce transgenic embryos in ovine, porcine, feline, bovine and equine species. Reprod. Fertil. Dev. 20:741-749.

Pozzobon, S. E., M. A. Lagares, D. S. Brum, F. G. Leivas and M. I. Rubin. 2005. Addition of recombinant human growth hormone to in vitro maturation medium of bovine oocytes. Reprod. Domest. Anim. 40:19-22.

Pribenszky, C., S. Matyas, P. Kovacs, E. Losonczi, J. Zadori and G. Vajta. 2010. Pregnancy achieved by transfer of a single blastocyst selected by time-lapse monitoring. Reprod. Biomed. Online. 21:533-536.

Purcell, S. H. and K. H. Moley. 2009. Glucose transporters in gametes and preimplantation embryos. Trends Endocrinol. Metab. 20:483-489.

Ramey, H. R., J. E. Decker, S. D. McKay, M. M. Rolf, R. D. Schnabel and J. F. Taylor. 2013. Detection of selective sweeps in cattle using genome-wide SNP data. BMC Genomics. 14:382-2164-14-382.

Rand, J. D. and C. M. Grant. 2006. The thioredoxin system protects ribosomes against stress-induced aggregation. Mol. Biol. Cell. 17:387-401.

Reed, M. L., A. Hamic, D. J. Thompson and C. L. Caperton. 2009. Continuous uninterrupted single medium culture without medium renewal versus sequential media culture: A sibling embryo study. Fertil. Steril. 92:1783-1786.

Rekik, W., I. Dufort and M. A. Sirard. 2011. Analysis of the gene expression pattern of bovine blastocysts at three stages of development. Mol. Reprod. Dev. 78:226-240.

Rizos, D., M. A. Ramirez, B. Pintado, P. Lonergan and A. Gutierrez-Adan. 2010. Culture of bovine embryos in intermediate host oviducts with emphasis on the isolated mouse oviduct. Theriogenology. 73:777-785.

Rosenkrans, C. F.,Jr and N. L. First. 1994. Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes in vitro. J. Anim. Sci. 72:434-437.

Saadeldin, I. M., B. Kim, B. Lee and G. Jang. 2011. Effect of different culture media on the temporal gene expression in the bovine developing embryos. Theriogenology. 75:995-1004.

Sanches, B., J. Pontes, A. Basso, C. Ferreira, F. Perecin and M. Seneda. 2013. Comparison of synthetic oviductal fluid and G1/G2 medium under low-1 oxygen atmosphere on embryo production and pregnancy rates in nelore (bos indicus) cattle. Reprod. Dom. Anim. 48:7-9.

Seli, E., C. Bruce, L. Botros, M. Henson, P. Roos, K. Judge, T. Hardarson, A. Ahlstrom, P. Harrison, M. Henman, K. Go, N. Acevedo, J. Siques, M. Tucker and D. Sakkas. 2011. Receiver operating characteristic (ROC) analysis of day 5 morphology grading and metabolomic viability score on predicting implantation outcome. J. Assist. Reprod. Genet. 28:137-144.

Senatore, E. M., J. Xu, M. V. Suarez Novoa, G. Gong, T. Lin, A. Bella, J. F. Moreno, M. E. Mannino, X. Tian, G. A. Presicce, S. C. Wu and F. Du. 2010. Improved in vitro development of OPU-derived bovine (bos taurus) embryos by group culture with agarose-embedded helper embryos. Theriogenology. 74:1643-1651.

Shi, L. Z., J. Nascimento, E. Botvinick, B. Durrant and M. W. Berns. 2011. An interdisciplinary systems approach to study sperm physiology and evolution. Wiley Interdiscip. Rev. Syst. Biol. Med. 3:36-47.

Shirazi, A., H. Nazari, E. Ahmadi, B. Heidari and N. Shams-Esfandabadi. 2009. Effect of culture system on survival rate of vitrified bovine embryos produced in vitro. Cryobiology. 59:285-290.

Smitz, J. E., J. G. Thompson and R. B. Gilchrist. 2011. The promise of in vitro maturation in assisted reproduction and fertility preservation. Semin. Reprod. Med. 29:24-37.

Solé, M., J. Santaló, I. Rodríguez, M. Boada, B. Coroleu, P. Barri and A. Veiga. 2011. Correlation between embryological factors and pregnancy rate: Development of an embryo score in a cryopreservation programme. J. Assist. Reprod. Genet. 28:129-136.

Somfai, T., Y. Inaba, Y. Aikawa, M. Ohtake, S. Kobayashi, T. Akai, H. Hattori, K. Konishi and K. Imai. 2010. Culture of bovine embryos in polyester mesh sections: The effect of pore size and oxygen tension on in vitro development. Reprod. Domest. Anim. 45:1104-1109.

Somfai, T., Y. Inaba, Y. Aikawa, M. Ohtake, S. Kobayashi, K. Konishi, T. Nagai and K. Imai. 2010. Development of bovine embryos cultured in CR1aa and IVD101 media using different oxygen tensions and culture systems. Acta Vet. Hung. 58:465-474.

Stamatkin, C. W., R. G. Roussev, M. Stout, C. B. Coulam, E. Triche, R. A. Godke and E. R. Barnea. 2011a. Preimplantation factor negates embryo toxicity and promotes embryo development in culture. Reprod. Biomed. Online. 23: 517-524.

Stamatkin, C. W., R. G. Roussev, M. Stout, V. Absalon-Medina, S. Ramu, C. Goodman, C. B. Coulam, R. O. Gilbert, R. A. Godke and E. R. Barnea. 2011b. PreImplantation factor (PIF) correlates with early mammalian embryo development-bovine and murine models. Reprod. Biol. Endocrinol. 9:63-73.

Sturmey, R. G., P. Bermejo-Alvarez, A. Gutierrez-Adan, D. Rizos, H. J. Leese and P. Lonergan. 2010. Amino acid metabolism of bovine blastocysts: A biomarker of sex and viability. Mol. Reprod. Dev. 77:285-296.

Sturmey, R., A. Reis, H. Leese and T. McEvoy. 2009. Role of fatty acids in energy provision during oocyte maturation and early embryo development. Reproduction in Domestic Animals. 44:50-58.

Sudano, M. J., D. M. Paschoal, S. Rascado Tda, L. C. Magalhaes, L. F. Crocomo, J. F. de Lima-Neto and C. Landim-Alvarenga Fda. 2011. Lipid content and apoptosis of in vitro-produced bovine embryos as determinants of susceptibility to vitrification. Theriogenology. 75:1211-1220.

Suarez SS, Regulation of sperm storage and movement in the mammalian oviduct. 2008. Int. J. Dev. Biol. 52: 455-462.

Sugimura, S., T. Akai, T. Somfai, M. Hirayama, Y. Aikawa, M. Ohtake, H. Hattori, S. Kobayashi, Y. Hashiyada, K. Konishi and K. Imai. 2010. Time-lapse cinematography-compatible polystyrene-based microwell culture system: A novel tool for tracking the development of individual bovine embryos. Biol. Reprod. 83:970-978.

Summers, M. C., P. R. Bhatnagar, J. A. Lawitts and J. D. Biggers. 1995. Fertilization in vitro of mouse ova from inbred and outbred strains: Complete preimplantation embryo development in glucose-supplemented KSOM. Biol. Reprod. 53:431-437.

Sun, Y. and B. Rigas. 2008. The thioredoxin system mediates redox-induced cell death in human colon cancer cells: Implications for the mechanism of action of anticancer agents. Cancer Res. 68:8269-8277.

Sutton-McDowall, M. L., R. B. Gilchrist and J. G. Thompson. 2010. The pivotal role of glucose metabolism in determining oocyte developmental competence. Reproduction. 139:685-695.

Sutton-McDowall, M. L., D. Feil, R. L. Robker, J. G. Thompson and K. R. Dunning. 2012. Utilization of endogenous fatty acid stores for energy production in bovine preimplantation embryos. Theriogenology. 77:1632-1641.

Swain, J. E. 2010. Optimizing the culture environment in the IVF laboratory: Impact of pH and buffer capacity on gamete and embryo quality. Reprod. Biomed. Online. 21:6-16.

Tagawa, M., S. Matoba, M. Narita, N. Saito, T. Nagai and K. Imai. 2008. Production of monozygotic twin calves using the blastomere separation technique and well of the well culture system. Theriogenology. 69:574-582.

Taka, M., H. Iwayama and Y. Fukui. 2005. Effect of the well of the well (WOW) system on in vitro culture for porcine embryos after intracytoplasmic sperm injection. J. Reprod. Dev. 51:533-537.

Takada, L., A. M. Junior, G. Z. Mingoti, J. C. Balieiro, J. Cipolla-Neto and L. A. Coelho. 2010. Effect of melatonin on DNA damage of bovine cumulus cells during in vitro maturation (IVM) and on in vitro embryo development. Res. Vet. Sci. 92: 124-127.

Tervit, H. R. and L. E. Rowson. 1974. Birth of lambs after culture of sheep ova in vitro for up to 6 days. J. Reprod. Fertil. 38:177-179.

Tervit, H. R., D. G. Whittingham and L. E. Rowson. 1972. Successful culture in vitro of sheep and cattle ova. J. Reprod. Fertil. 30:493-497.

Thompson, J. G. 2000. In vitro culture and embryo metabolism of cattle and sheep embryos - a decade of achievement. Anim. Reprod. Sci. 60-61:263-275.

Thompson, J. G., C. McNaughton, B. Gasparrini, L. T. McGowan and H. R. Tervit. 2000. Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured in vitro. J. Reprod. Fertil. 118:47-55.

Thompson, J. G. 2007. Culture without the petri-dish. Theriogenology. 67:16-20.

Trigal, B., E. Gomez, C. Diez, J. N. Caamano, D. Martin, S. Carrocera and M. Munoz. 2011. In vitro development of bovine embryos cultured with activin A. Theriogenology. 75:584-588.

Ulbrich, S. E., K. Zitta, S. Hiendleder and E. Wolf. 2010. In vitro systems for intercepting early embryo-maternal cross-talk in the bovine oviduct. Theriogenology. 73:802-816.

van der Valk, J., D. Brunner, K. De Smet, A. Fex Svenningsen, P. Honegger, L. E. Knudsen, T. Lindl, J. Noraberg, A. Price, M. L. Scarino and G. Gstraunthaler. 2010. Optimization of chemically defined cell culture media--replacing fetal bovine serum in mammalian in vitro methods. Toxicol. In Vitro. 24:1053-1063.

Velazquez, M. A., D. Hermann, W. A. Kues and H. Niemann. 2011. Increased apoptosis in bovine blastocysts exposed to high levels of IGF1 is not associated with downregulation of the IGF1 receptor. Reproduction. 141:91-103.

Velazquez, M. A., L. J. Spicer and D. C. Wathes. 2008. The role of endocrine insulin-like growth factor-I (IGF-I) in female bovine reproduction. Domest. Anim. Endocrinol. 35:325-342.

Vutyavanich, T., U. Saeng-Anan, S. Sirisukkasem and W. Piromlertamorn. 2011. Effect of embryo density and microdrop volume on the blastocyst development of mouse two-cell embryos. Fertil. Steril. 95:1435-1439.

Wan, P., Z. Hao, P. Zhou, Y. Wu, L. Yang, M. Cui, S. Liu and S. Zeng. 2009. Effects of SOF and CR1 media on developmental competence and cell apoptosis of ovine in vitro fertilization embryos. Anim. Reprod. Sci. 114:279-288.

Wang, Y. A., G. Kovacs and E. A. Sullivan. 2010. Transfer of a selected single blastocyst optimizes the chance of a healthy term baby: A retrospective population based study in australia 2004-2007. Hum. Reprod. 25:1996-2005.

Weiss, L., R. Or, R. C. Jones, R. Amunugama, L. JeBailey, S. Ramu, S. A. Bernstein, Z. Yekhtin, O. Almogi-Hazan, R. Shainer, A. O. Vortmeyer, M. J. Paidas, M. Zeira, S. Slavin and E. R. Barnea. 2012. Preimplantation factor (PIF*) reverses neuroinflamation while promoting neural repair in EAE model. J. Neurol. Sci. 15: 146-157.

Wiltbank, M. C., R. Sartori, M. M. Herlihy, J. L. Vasconcelos, A. B. Nascimento, A. H. Souza, H. Ayres, A. P. Cunha, A. Keskin, J. N. Guenther and A. Gumen. 2011. Managing the dominant follicle in lactating dairy cows. Theriogenology. 76:1568-1582.

Yanagimachi, R. and M. C. Chang. 1964. In vitro fertilization of golden hamster ova. J. Exp. Zool. 156:361-375.

Yoshioka, K., M. Noguchi and C. Suzuki. 2012. Production of piglets from in vitro-produced embryos following non-surgical transfer. Anim. Reprod. Sci. 131:23-29.

Zhang, M., Y. Q. Su, K. Sugiura, K. Wigglesworth, G. Xia and J. J. Eppig. 2011. Estradiol promotes and maintains cumulus cell expression of natriuretic peptide receptor 2 (NPR2) and meiotic arrest in mouse oocytes in vitro. Endocrinology. 152:4377-4385.

TABLES

Table 1. Important milestones in embryo culture media.

Event	Reference
-First IVF reported using the golden hamster model.	Yanagimachi and Chang (1964).
-Tervit et al. developed a synthetic oviduct media based on the biochemistry and physiology of the ovine oviduct.	Tervit et al. (1973).
-Bavister and co-workers did intensive work during the 70's to meet in vitro embryo nutrient requirements.	Bavister et al. (1983).
-Biggers and co-workers created KSOM and 2-cell block in mice was overcome	Summers et al. (1995).

Table 2. Past and current focus on embryo metabolism in livestock species.

Metabolic area	Factor studied*	Reference
-Modulation of glucose consumption at precompaction stage	EDTA	Reviewed in Thompson et al. (2000).
-Enhancement of glucose consumption at pericompaction stages	DNP	Thompson et al. (2000).
	PES	De La Torre-Sanchez et al. (2006).
	HIF	Harvey (2007).
-Utilization of internal lipid stores	L-Carnitine	Sturmey et al. (2009). Sutton-McDowall et al. (2012).
-Progesterone-related nutrient metabolism	AA +Glucose	Clemente et al. (2009). Hugentobler et al. (2010). Garcia-Herreros et al. (2012).
-Sex-dependent nutrient requirements	AA	Sturmey et al. (2010).
	Glucose	Garcia-Herreros et al. (2012.

*Abbreviations: EDTA (ethylenediaminetetraacetic acid), DNP (2, 4—Dinitro Phenol), PES (Phenazine ethosulfate), HIF (Hypoxia inducible factors) and AA (Amino acids).

Table 3. Consensus average embryo blastocyst rates among large animal domestic species cultured with different media formulations.

Media type	Species	In vitro procedure	Blastocyst rate (%)	Reference
KSOM	Porcine	IVF	32.1	Hashem et al. (2006)
	Bovine	IVF	37.1	Felmer et al. (2011)
SOFaa	Porcine	Cloning	38.0[a]	Lagutina et al. (2006)
	Ovine	IVF	24.1	Garcia-Garcia et al. (2007)
	Ovine	IVF	28.7	Wan et al. (2009)
	Bovine	IVF	34.9	Sanches et al. (2013)
CR1aa	Equine	ICSI	25.5[b]	Matsukawa et al. (2007)
	Ovine	IVF	25.5	Wan et al. (2009)
	Bovine	IVF	36.1	Somfai et al. (2010)
DMEM/F-12	Equine	ICSI	42.0[c]	Hinrichs. (2012)
NCSU-23	Porcine	IVF	30.5	Hashem et al. (2006)
	Porcine	Cloning	28.5[a]	Lagutina et al. (2006)
	Porcine	IVF	32.8	Kim et al. (2012)
PZM	Porcine	IVF	27.4[d]	Hu et al. (2012)
	Porcine	IVF	27.5[e]	Yoshioka et al. (2012)
IVD101	Bovine	IVF	37.1	Hoshi (2003)
	Bovine	IVF	21.7	Somfai et al. (2010)
Gardner (G1/G2)	Ovine	IVF	21.5	Garcia-Garcia et al. (2007)
	Bovine	IVF	27.8	Sanches et al. (2013)
IVF VET Solutions	Bovine	IVF	69.0[f]	Albuz et al. (2010)

[a]Overall average blastocyst rate from 3 different donor cell lines.

[b]Blastocyst rates originated from expanded oocytes.

[c]Highest blastocyst rate reported for equine ICSI-produced embryos.

[d]Blastocyst rate achieved by supplementing Vitamin C (20µg/ml).

[e]Blastocyst rate reported at day 5 post IVF.

[f]High blastocyst rate achieved by a novel approach, stimulated physiological oocyte maturation (SPOM), during IVM.

Table 4. Supplementation with embryotrophic factors towards improving (more) defined media.

Factor	Actions	Reference
Colony-stimulating factor 2 (CSF2)	Blocked apoptosis in bovine embryos through regulatory actions on apoptosis genes. CSF2-embryos tended to secrete more IFNτ.	Loureiro et al., 2011a and b
Epidermal growth factor (EGF)	Besides its role on proliferation, migration and differentiation, EGF is involved in reduction of apoptosis during preimplantation and placentation. Higher cryotolerance has been reported in bovine embryos treated with EGF at IVM.	Gordon, 2003; Hambruch et al., 2010; Dhali et al., 2011
Fibroblast growth factor 2 (FGF2)	FGF2, but more importantly FGF2 receptor activity were necessary for optimal development	Fields et al., 2011
Preimplantation factor (PIF)	Involved in modulation of local immunity, promoting decidual proadhesion molecules and enhancement of trophoblast invasion. In addition, PIF was found to be expressed in the trophoblast layer during the first two trimesters of human pregnancy.	Stamatkin et al., 2011a and b; Weiss et al., 2012; Barnea and Sharma. 2006
Insulin, transferrin and sodium selenite (ITS)	Insulin exerted its actions on glucose transport and AA uptake along with antioxidant protection from transferrin and selenium.	Gordon, 2003
GH (somatotropin) and IGF-I	In combination stimulated embryonic development. Although the GH effects have been reported as not mediated by IGF-I in the bovine IVM model, both hormones appear to have critical roles in the modulation of apoptosis and metabolism. Embryonic development is stimulated by the combination.	Moreira et al. 2002; Bevers and Izadyar, 2002; Kölle et al., 2002; Köle et al., 2004; Pozzobon et al., 2005; Kuzmina et al., 2007; Velazquez et al., 2008; Velazquez et al., 2011 Makarevich et al., 2012
IGF-I, IGF-II, FGF2, transforming growth factor beta1 ($TGF_{\beta 1}$), leukemia inhibitory factor (LIF) and CSF2, singly and in combination.	Results from the combination were similar to culture under serum conditions, but not greater.	Neira et al. 2011
Activin A	In bovine species, fourth cell cycle (lag phase) is longer for in vitro derived embryos than in vivo counterparts (~50 hr vs. ~24 hr). Use of activin A shortened the lag phase and was also beneficial at later (5-8 days post insemination [dpi]) stages, however, more rapid rates of development were associated with higher rates of apoptosis.	Gordon, 2003; Trigal et al. 2011
Thyroid hormone (TH)	Important during early pregnancy but the use in vitro is minimal. On the bovine embryo TH is involved in lowering apoptotic rates as well as improved hatching rates post thawing for cryopreserved embryos.	Ashkar et al., 2010

Table 5. The use and benefits of alternative macromolecules in bovine embryo culture systems.

Macromolecule	Culture	Main findings	Reference
rHSA + HA	Group	Inclusion of both macromolecules plus citrate resulted in high rates of bovine blastocyst development.	Lane et al., 2003.
BSA + HA	Group	The addition of BSA was necessary throughout the entire culture period and resulted in higher blastocyst rates irrespective of HA treatment. However, supplementation of HA at day 4 post insemination increased mitochondrial DNA (mtDNA) copy numbers at the blastocyst stage.	Palasz et al., 2009.
KOSR	Group	KOSR at 20% O_2 yielded the highest pre-implantation embryo rates.	Mingoti et al., 2011.
human embryonic stem cell derived bioactive material (hES-BM)	Group	Results from staining methods and measures of gene expression of pluripotency, embryogenesis and growth markers were significantly higher than in the control group treated with fetal bovine serum. The authors concluded that hES-BM could be used as a new supplement for bovine preimplantation embryo development.	Kim et al., 2011.
Agarose-embedded helper embryos	Group (co-culture)	Enhanced developmental competence and embryo quality markers e.g. involved in implantation and normal calf delivery (PGHS2, TXN, PLAU).	Senatore et al., 2010; Deb et al., 2011.
Somatic cell feeders	Group	Cryotolerance was positively influenced by the presence of somatic cells and was reflected in survival and hatching rates as well as differential cell counts.	Shirazi et al., 2009.
Heterologous cumulus cells	Single (co-culture)	Gene expression for qualitative markers (TP53, BAX, SHC1, SHC and IGF2R) was reportedly similar for groups of embryos co-cultured singly or in groups with or without heterologous cumulus cells.	Goovaerts et al., 2011.

FIGURES

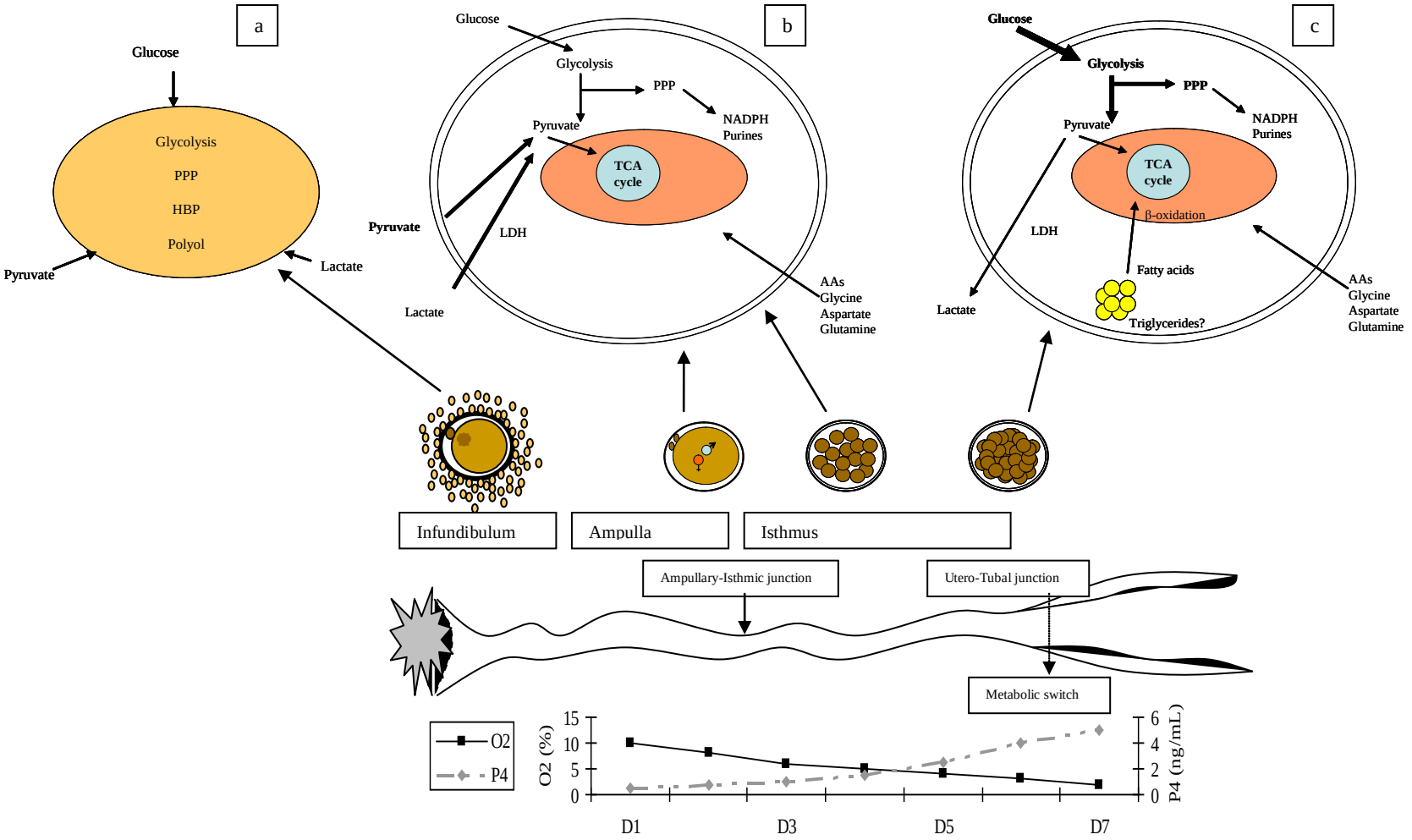
a
Glucose
Glycolysis
PPP
HBP
Polyol
Pyruvate
Lactate
b
Glucose
Glycolysis
PPP
NADPH
Purines
Pyruvate
TCA cycle
Pyruvate
LDH
Lactate
AAs
Glycine
Aspartate
Glutamine
c
Glucose
Glycolysis
PPP
NADPH
Purines
Pyruvate
TCA cycle
β-oxidation
LDH
Fatty acids
Lactate
Triglycerides?
AAs
Glycine
Aspartate
Glutamine
Infundibulum
Ampulla
Isthmus
Ampullary-Isthmic junction
Utero-Tubal junction
Metabolic switch
O2
P4
O2 (%)
P4 (ng/mL)
15
10
5
0
6
4
2
0
D1
D3
D5
D7

Figure 1. Summary of embryo metabolic events during preimplantation.

a) Within the cumulus oocyte complex, the oocyte emphasizes glycolysis and pentose phosphate (PPP) pathways. Glycolysis provides an important source of energy via hexokinase activity. PPP provides reducing equivalents (NADPH) and production of pentoses for the synthesis of purines. The hexosamine biosynthesis pathway (HBP) is important for extracellular matrix expansion of the cumulus cells and o-linked glycosylation of important amino acids residues involved in the cell cycle progression. Polyol pathway provides sorbitol and fructose as other sources of energy and it contributes to maintenance of redox balance. b) After fertilization, the zygote depends on tricarboxylic acids such as pyruvate and lactate as the primary energy sources. c) Bovine morula compaction is an important event that occurs at day five after fertilization in the bovine embryo and marks the beginning for higher glucose consumption. Thus, in preparation for a hypoxic environment in the uterus and coincident with the increase in plasma progesterone (P4), the preimplantation embryo at morula stage switches its metabolism from low to high glucose utilization via glycolysis and PPP pathways.

CHAPTER THREE: EFFECT OF CONJUGATED LINOLEIC ACID SUPPLEMENTATION ON *IN VITRO* BOVINE EMBRYO PRODUCTION AND CRYOPRESERVATION

V. A. Absalón-Medina[†], S. J. Bedford-Guaus[‡], R. O. Gilbert[‡], L. C. Siqueira[1], G. Esposito[2], A. Schneider[3], S. H. Cheong[‡], and W. R. Butler[†*]

Submitted, Journal of Dairy Science

[†]College of Agriculture and Life Sciences, Animal Science Department. Cornell University, Ithaca, NY 14853. USA.

[‡]College of Veterinary Medicine, Department of Clinical Sciences. Cornell University, Ithaca, NY 14853. USA

[1]Universidade Federal de Santa Maria, Santa Maria, RS 8690. Brazil.

Email: lsvet@bol.com.br

[2]Università degli Studi di Napoli Federico II, Portici, Napoli 80138. Italy.

Email: Giulia.Esposito@up.ac.za

[3]Universidade Federal de Pelotas, Pelotas, RS 96001. Brazil.

Email: augustoschneider@gmail.com

[*]Correspondence e-mail: wrb2@cornell.edu

ABSTRACT

Conjugated linoleic acid (CLA) isomers of linoleic acid (18:2 n-6) and other polyunsaturated fatty acids can affect the lipid profile and signaling of cells and thereby alter their function. A total of 6,267 bovine oocytes were used in this project in a structured series of experiments to determine the optimal isomer dose and stage of development for supplementation with CLA, *cis* 9, *trans* 11 and *trans* 10, *cis* 12, to improve in vitro embryo production. In experiment 1, high doses of CLA during in vitro maturation (IVM) were compared with high or low doses during the entire in vitro embryo culture of parthenogenetic embryos. High and low doses of CLA ranged from 50 to 200 μM and from 15 to 50 μM, respectively. In experiment 2 low doses of CLA were tested during the entire culture after in vitro fertilization. Experiment 3 studied the effect of low doses of CLA at specific points during in vitro fertilization and embryo culture. In experiment 4, resistance to cryopreservation *viz.* vitrification of embryos supplemented with CLA was assessed by post-thaw survival rates. In experiment 1 there were no effects ($p > 0.05$) of CLA on maturation or cleavage rates (92.2 ± 1.6 % and 78.3 ± 4.1 %, respectively). When CLA was provided at maturation only, blastocyst rates remained similar across treatments averaging 28.9 ± 5.1 %. However, high doses of CLA during the entire embryo culture period decreased ($p < 0.05$) blastocyst rates (7-15%) in a dose dependent manner. Although no differences in blastocyst rates were observed using low doses of CLA during the entire culture, these rates were lower (18.4 ± 1.9 %) compared to groups treated with CLA at maturation only. Progesterone concentrations in maturation media were significantly higher for

the three high dose treatment groups combined i.e. 50 μM, 100 μM and 200 μM as compared to control (0.77 ± 0.09 ng/ml vs. 0.55 ± 0.09 ng/ml; $p < 0.05$). Low doses of CLAs (15 μM to 50 μM) in IVM media did not affect progesterone concentrations. In experiment 2, no differences were observed among treatment groups in cleavage rates (average 84.9 ± 1.9 %) and blastocyst rates in response to CLA were generally similar to control (38.2 ± 2.3 %). In experiment 3, low doses of either CLA isomer during the entire IVC did not affect cleavage rates (89.6 ± 1.6 %), however, blastocyst rates were significantly reduced for *t*10,*c*12 CLA and *c*9, *t*11 when compared to control (24.6 ± 1.8 % and 30.8 ± 1.4 % vs. 34.18 ± 1.2 %, respectively) and the negative effect ($p < 0.05$) was enhanced by time in culture, especially in the presence of *t*10, *c*12 CLA. In experiment 4, vitrifying embryos supplemented with a high dose of CLA *c*9, *t*11 during the final 36 hr of IVC resulted in the highest survival rates and the quality of thawed embryos was comparable to control embryos not undergoing vitrification. In conclusion, CLA supplementation did not improve embryo production, but inclusion of CLA *c*9, *t*11 in vitrification protocols improved post-thaw survival of bovine embryos produced in vitro.

INTRODUCTION

Conjugated linoleic acid (CLA) comprises a group of polyunsaturated fatty acids (PUFA) derived from linoleic acid (C18:2n-6) during biohydrogenation by ruminal flora in cattle and sheep. CLA is also produced from delta-9 desaturation of vaccenic acid (*trans* 11 18:1; Bougnoux et al., 2009). Between the two CLA isomers, *cis*9, *trans*11 (*c*9, *t*11) is substantially more abundant than *trans*10, *cis*12 (*t*10, *c*12; Lock and Bauman, 2004; Bauman et al., 2008). These CLA isomers and other PUFAs can affect the membrane lipid profile and signaling of cells thereby altering their function in vivo or in vitro (Pereira et al., 2008; Fouladi-Nashta et al., 2009; Marei et al., 2009; Al Darwich et al., 2010). Regarding changes in lipid composition, CLAs become relevant for the improvement of some assisted reproduction technologies such as cryopreservation of gametes and embryos (Pereira et al., 2007; Pereira et al., 2008; Pereira and Marques, 2008). Embryos may suffer considerable morphological and functional damage and the extent of the injury as well as differences in post thaw performance may be highly variable depending on the developmental stage and origin (i.e. in vitro produced or in vivo derived embryos). Currently, there are two methods of choice for oocyte and embryo cryopreservation: slow freezing or vitrification, of which the latter seems to yield better results when working with relatively low numbers of embryos (Pereira and Marques, 2008). Cytoplasmic lipid content and lipid composition of membranes are of concern for *in vitro* embryo production since they can be affected by culture media in ways that increase their vulnerability to damage during cryopreservation procedures.

In regard to media composition, supplementation with serum may yield higher blastocyst rates and improve overall embryonic performance; however, serum supplementation also increases cytoplasm lipid uptake and lowers phosphatidyl choline and cholesterol esters (Pereira et al., 2007; Al Darwich et al., 2010). Conversely, when serum-containing embryo culture medium was supplemented with CLA *t*10, *c*12, lipid droplet formation was reduced (Pereira et al., 2008). The authors proposed that embryo incorporation of CLA *t*10, *c*12 during culture increased membrane fluidity thus conferring embryos with a greater resistance to cryopreservation-induced damage. Moreover, it was suggested that CLA *t*10, *c*12 inhibited stearoyl-CoA desaturase (SCD1; delta-9 desaturase) to make embryos less vulnerable to chilling stress (Al Darwich et al., 2010).

In addition to their purported effect in cell membrane function, some fatty acids may play roles in cholesterol metabolism and thus contribute in the regulation of steroidogenesis (Fouladi-Nashta et al., 2009). Along these lines, there is a current debate as to the effect of progesterone secretion by cumulus cells during oocyte maturation. While some studies report that progesterone produced during this time period negatively affects later embryo development, others suggest that this hormone is involved in the acquisition of oocyte competence and regulation of cumulus-cell apoptosis (reviewed by Fair and Lonergan, 2012). The different results among these studies may stem from different culture systems or different PUFAs.

Given the conflicting results among studies, the effect of CLA supplementation during in vitro culture of bovine oocytes and embryos requires systematic investigation. In the current study, we evaluated the effects of supplementing culture

medium with varying concentrations of two CLA isomers for their effects upon bovine oocytes, parthenotes and preimplantation embryos. In addition, the effects of CLA supplementation upon post-thaw survival of vitrified embryos were also assessed.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents were obtained from Sigma-Aldrich Co. (Saint Louis, MO, USA), unless otherwise stated.

Experimental design

Experiment 1: Effects of CLA supplementation before and after parthenogenetic activation of bovine oocytes on embryonic development.

The aims of this experiment were to compare the effect of high-dose CLA supplementation in maturation medium only with high or low-dose CLA supplementation throughout the entire culture period. Varying dose ranges were based on Pereira et al. (2008) and they were tested for each CLA isomer (*c*9, *t*11 and *t*10, *c*12) where 50, 100, and 200 μM doses were considered 'High' and 15, 25, and 50 μM doses were considered 'Low'. For all CLA concentrations, osmolarity was similar among CLA groups with carrier bovine serum albumin added to each control group and no mineral oil overlay was used. This experiment was divided in three parts starting with the evaluation of CLA supplementation during IVM (N= 1062 oocytes), high dose CLA supplementation throughout oocyte and embryo culture (N=1756 oocytes), and low dose CLA-supplementation also throughout (N=1206 oocytes),

respectively. Each experiment was performed at least in triplicate. Following oocyte removal, in vitro maturation (IVM) media from each treatment group was stored at -20 °C for progesterone analysis.

Experiment 2: Effect of low-dose CLA supplementation throughout the entire culture period before and after in vitro fertilization (IVF).

In four replicates a total of 839 oocytes were matured, fertilized and the corresponding embryos cultured in vitro. Oocytes were placed in media supplemented with CLA or carrier control for the entire in vitro culture period including IVM. Treatment groups were a 15 and 25 μM doses for each CLA isomer (*c*9, *t*11 or *t*10, *c*12) and the corresponding carrier controls. Variables measured included cleavage and blastocyst formation rates, as well as the neutral lipid (hereafter lipid) content of embryos at d-8 post fertilization as assessed by Nile red fluorescent staining under an epifluorescent microscope (Bonilla and Hansen, 2009). For all the experiments involving staining, fixed embryos were mounted on gold seal fluorescent antibody microslides with two etched 10 mm diameter circles surrounded by white ceramic ink (Thermo Scientific, USA). Expression of the embryonic survival markers *survivin* and *HSP 70.1* genes was measured on d-8 after IVF based on Rief et al. (2002) and Rizos et al. (2004).

Experiment 3: Effect of low-dose CLA supplementation during oocyte maturation only, or during IVF and embryo culture process.

For this trial, the effects of a low (15 μM) dose of the tested CLA isomers were evaluated either during IVM only, or during and after IVF. Nine hundred and two oocytes were used in four replicates to determine the embryonic viability at d-8 post

fertilization. Variables measured were cleavage and blastocyst rates as well as the expression of *survivin* and *HSP 70.1* genes. Total blastomere count was assessed using Hoechst 33342 staining (Bonilla and Hansen, 2009).

Experiment 4: Effect of CLA supplementation on embryo viability following vitrification and thawing.

For this experiment 502 oocytes were used across 6 groups including control, CLA *c*9, *t*11 (15 μM) , CLA *t*10, *c*12 (15 μM), CLA *c*9, *t*11 (100 μM) and CLA *t*10, *c*12 (100 μM). The final group was a quality control without CLA supplementation and not undergoing vitrification procedures (Control+). Further, groups received 15 μM CLAs dose only during IVM and remained CLA-free throughout subsequent culture. The CLA treatment for the 2 groups with 100 μM of each isomer consisted of adding the CLAs for 36 hrs prior to vitrification at d-6.5 post-fertilization. Embryos (mostly morula stage) were vitrified (BoviPRO Vit Kit^{TM} from Minitube) and subsequently thawed according to the manufacturers' instructions. Immediately after vitrification of all embryos were thawed, incubated for 48 hr, fixed, and stained with Hoechst 33342. Variables measured included cleavage, blastocyst and post thawing blastocyst-survival rates, as well as total blastomere cell counts during survival assessment.

Oocyte recovery and selection

Bovine ovaries were collected at a nearby abattoir (120 km) and transported to the laboratory in prewarmed (30-35 °C) lactated Ringer's solution at. Cumulus-oocyte complexes (COCs) from 2-8 mm follicles were aspirated with an 18G hypodermic

needle attached to an aspiration pump unit adjusted to a pressure of 22.5 ± 2.5 ml of H_2O per minute. Follicular fluid supernatant was removed and the pellet containing COCs was transferred to a 15 ml tube where it was resuspended with holding medium and contents were poured gently into a 100 mm Petri dish. Holding medium consisted of TCM-199 Hank's salts (Invitrogen, Grand Island, NY) supplemented with 10% Fetal Calf Serum (FCS; Invitrogen), 25 μg/ml of gentamicin, 5 μg/ml heparin, at pH 7.35 ± 0.02 and mOsm = 300 ± 1. After morphological assessment of COCs, only those with several layers of granulosa cumulus cells and oocytes with a homogenous cytoplasm were selected for use. The entire process was performed within 5 hours including transportation.

In vitro maturation

Selected COCs were matured in groups of 40 ± 5 for 23 ± 1 hours in 400 μL of TCM-199 (Earle's Salts) enriched with 10% FCS, 22 μg/ml sodium pyruvate, 1 mM alanyl-glutamine, 0.1 mM taurine, 0.1 mM cysteamine, 1 μg/ml estradiol, 5 μg/ml luteinizing hormone (LH; SIOUX Biochemical, Inc., Sioux Center, IA), 0.5 μg/ml follicle stimulating hormone (FSH; SIOUX), 10 ng/ml epidermal growth factor (EGF; BD Biosciences—Discovery Labware, MA), 20 μg/ml gentamicin, at pH 7.35 ± 0.02 and mOsm 300 ± 2, and covered with light mineral oil in a humidified atmosphere at 38.5° C with 5% CO_2 in air.

Parthenogenetic oocyte activation and embryo culture after maturation

Parthenotes were obtained following the protocol used by Bastos et al. (2008). Briefly, groups of COCs were placed into a small petri dish containing 0.2 mg of

hyaluronidase per ml of holding media (pH 7.35 ± 0.02, mOsm 300 ± 2) for 3 min and then transferred into a 1.8 ml centrifuge tube. Contents were vortexed at maximum speed for 5 min to remove the cumulus cells. Cumulus-free oocytes were then resuspended in holding medium and, by fine glass pipette rotation, only those presenting a visible polar body indicating successful progression of nuclear maturation to the metaphase II stage (MII) were selected and used for parthenogenetic activation (PA). Then, MII oocytes were exposed to 5 μM ionomycin for 5 min in M-199 (Hank's Salts; Invitrogen, Grand Island, NY) medium supplemented with 0.4% (w/v) BSA, rinsed three times in the same medium without ionomycin, and then incubated for 5 hr in modified synthetic oviduct fluid medium (SOF, pH 7.4 ± 0.01, mOsm 275 ± 5; Holm et al., 1999) containing 2.8 mM 6-dimethylaminopurine (6-DMAP; Susko-Parrish et al.,1994). After this short incubation treatment, oocytes were rinsed twice and cultured in SOF medium supplemented with 0.4% (w/v) BSA and both essential and nonessential amino acids at 38.5° C in 5% CO_2, 5% O_2 and 90% N_2. Rates of cleavage were evaluated at 48 hr after activation. After cleavage assessment, PA embryos were cultured in groups of ten in 100 μl SOF droplets and transferred every two days to fresh medium. Blastocyst rates were assessed at d-8 after activation.

In vitro fertilization

Presumptive matured oocytes were transferred to a modified IVF medium (Fert-TALP; Parrish et al., 1988) supplemented with 0.5 mM fructose, 6 mg/ml BSA FFA Fraction V, 30 μM penicillamine, 15 μM hypotaurine, 1.5 μM epinephrine (PHE), 22 μg/ml heparin, 20 μg/ml gentamicin, pH of 7.38 ± 0.01, mOsm 290 ± 2 and

covered with light mineral oil in a humidified atmosphere at 38.5° C with 5% CO_2 in air for 18-22 hours. Frozen semen straws (Genex, Ithaca NY, USA) from a single bull and the same ejaculate were used for the 3 IVF experiments, following thawing at 37° C for 30 seconds for each trial. Motile sperm were separated from non-motile ones, cryoprotectant and debris by Percoll double density gradient (90, 45%) centrifugation at 700 x g for 20 min. Subsequently, sperm were washed twice in 5 ml of TL-Semen (pH 7.39 ± 0.01, mOsm 295 ± 2) modified Tyrode's from Parrish et al. (1989) at 300 x g for 5 min to remove Percoll. Finally, sperm were adjusted to a final concentration of 1.5 x 10^6 sperm/ml using Fert-TALP media; in all cases progressive motility averaged at least 50%.

In vitro embryo culture

A modified SOF medium (Holm et al., 1999) was used for in vitro embryo culture (20 μg/ml gentamicin, pH 7.4 ± 0.01, mOsm 275 ± 5). After fertilization, putative zygotes were denuded at maximum vortex speed for 120 sec and transferred to a modified SOF (SOF_{early}) supplemented with 10 μM EDTA, 0.5 mM fructose, 0.4 % (w/v) BSA FFA Fraction V, 0.1 mM taurine, and then placed in light mineral oil-covered droplets in a humidified atmosphere at 38.5° C with 5% CO_2, 5% O_2, and 90% N_2. Following ~48 hr in culture, cleavage rates were assessed and embryos were transferred to new droplets containing SOF_{mid}, with essential and non essential amino acids, 0.4 % (w/v) BSA FFA Fraction V, and 0.5 mM glucose in a humidified atmosphere at 38.5° C with 5% CO_2, 5% O_2, and 90% N_2. After ~96 hr embryos were again transferred to fresh SOF_{mid} droplets under the same conditions. Finally, d-7

embryos were transferred for the last ~ 24 hr of culture to SOF_{late}, which was SOF supplemented with 5% (v/v) FCS, 0.1 mM taurine and 0.5 mM glucose in a humidified atmosphere at 38.5° C with 5% CO_2, and 5% O_2, and 90% N_2. Blastocyst rates were calculated as percentages of the original oocyte number.

BSA:CLA-Complex

Isomers of CLA, *cis*9, *trans*11 and *trans*10, *cis*12 (> 90% pure), were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA). The CLA isomers were conjugated to fatty acid-free BSA in a 4:1 ratio to prepare 4.5 mM CLA-BSA stocks using a method adapted from Keating et al. (2006). Subsequent dilutions were made in culture media.

RNA isolation and transcript quantification

For total RNA extraction, 100 µL of Qiazol (Qiagen Valencia, CA, USA) was added to a known number (13 ± 4) of embryos 8-d after fertilization, vortexed and stored at -80^{o}C. Total RNA was isolated and purified using the RNeasy Mini columns (Qiagen Valencia, CA, USA) and on-column RNase-free DNase treatment (Qiagen Valencia, CA, USA) following the manufacturer's instructions. Then, RNA was eluted in a final volume of 15 µL. The reverse transcription reactions were performed using a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems Foster City, CA, USA) in a final volume of 20 µL. Real-time quantitative PCR was performed using SYBR Green dye to evaluate RPS9 (reference gene), *survivin,* and Hsp 70.1 gene expression with primers listed in Table 1. The amount of cDNA in each reaction was standardized to 1 embryo per reaction. The PCR reactions

were performed in duplicate in a 25-µL final volume using Power SYBR Green Mastermix (Applied Biosystems, Foster City, CA, USA). The PCR reactions and fluorescence detection were performed in the ABI Prism 7300 Sequence Detector (Applied Biosystems Foster City, CA, USA). For each assay, 40 PCR cycles were run and a dissociation curve was included to verify the amplification of a single PCR product. Analyses of amplification plots were performed with the Sequence Detection Software (Applied Biosystems, Foster City, CA, USA) to determine the counts for each sample. Each assay plate contained negative controls and a standard curve with 5 diluted liver samples (serial dilutions with known RNA concentrations) to determine amplification efficiency of the respective primer pair. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), RSP9 as a reference gene, and the control group to correct the fold changes. The average coefficient of variation was 1.20%.

Image acquisition of stained embryos

All slides were examined using an epifluorescent microscope (Imager Z1; Carl Zeiss, Inc.) under a 20× 0.5 NA ECPlan Neofluar (Carl Zeiss, Inc.). Embryo samples were excited at 340 nm and 488 nm with emission of 470 nm and 515 nm, to visualize DAPI nuclear stain and Nile red, respectively. Images were captured with a cooled charged-coupled device camera (AxioCam MRm; Carl Zeiss, Inc.) and processed using AxioVision software (version 4.7.2; Carl Zeiss, Inc.). Mean pixel intensity is reported as arbitrary units.

Progesterone radioimmunoassay

Progesterone concentrations in IVM media samples were quantified by radioimmunoassay (Elrod and Butler, 1993) with the addition of control media to standards. Inter and intra-assay coefficients of variation were 5.35% and 5.54%, respectively.

Statistical analysis

One-way analysis of variance (ANOVA) between treatments was performed using JMP version 10 (Statistical Discovery from SAS). If replicates were different overtime, we included replicate in our models as a random variable to control for the differences between and within experiments. If the main effect was significant all means were compared using Tukey HSD. A probability of $P < 0.05$ was considered statistically significant and $P \leq 0.1$ was considered a trend. Proportional data not being normally distributed was arc-sine transformed and results are presented in the tables as back transformed data. Likewise, numerical data not being normally distributed was transformed to its natural logarithm.

RESULTS

Experiment 1: Effect of CLA supplementation in vitro before and after parthenogenetic activation for embryonic development.

The effect of high dose CLA supplementation in maturation media only

Supplementation with CLA during IVM did not significantly ($P > 0.05$) affect the rate of oocyte maturation or the proportion of cleaved embryos and blastocysts at any dose level of either isomer (Table 2). Progesterone analyses of IVM media showed increased levels of progesterone in response to high doses of CLA and this effect was directly proportional to the CLA concentration (main effect $P < 0.05$; Figure 1). Conversely, low dose CLA treatment yielded increased progesterone levels only for the 50 μM concentration of *t*10, *c*12 (Figure 2).

Effect of high dose CLA supplementation throughout culture

Results of high dose CLA supplementation (50, 100 and 200 μM for each isomer) throughout culture are summarized in Table 3. At 200 μM, CLA *t*10, *c*12 reduced maturation rates as compared to the control group ($P < 0.05$). Conversely, supplementation with 50 μM CLA *t*10, *c*12 improved cleavage rates relative to control in the group ($P < 0.05$). Blastocyst rates were depressed by both CLA isomers in a dose dependent manner ($P < 0.05$).

The effect of low dose CLA supplementation throughout culture

The effects of low doses of CLA supplementation (15, 25 and 50 μM) for the entire culture period are presented in Table 4. No significant differences were

observed between groups for maturation rates. The group supplemented with CLA *t*10, *c*12 at 15 µM showed the highest cleavage rate (86.57%) which was higher ($P < 0.05$) than that for the control group. Conversely no statistical differences were detected among groups for blastocyst rates, although 50 µM CLA *t*10, *c*12 tended to depress blastocyst rate relative to the control group ($P = 0.08$).

Experiment 2: Effect of low-dose CLA supplementation throughout culture before and after in vitro fertilization.

Herein we tested the lowest concentrations of both CLA isomers from experiment 1 (Table 5). No differences were observed in cleavage rates. However, blastocyst rates were significantly depressed by 25 µM CLA *t*10, *c*12 supplementation. Then, the lipid content of d-8 embryos was determined by epifluorescence microscopy after Nile red staining (Figure 3). Lipid content was increased by the CLA *t*10, *c*12 isomer when compared to the control group ($P < 0.05$), and treatment with CLA *c*9, *t*11 had a significant effect only when provided at 25 µM. Relative expression of the *survivin* gene was depressed by 15 and 25 µM of CLA *t*10, *c*12 and by 15 µM CLA *c*9, *t*11 (Figure 4). Expression of HSP 70.1 was decreased only by CLA *t*10, *c*12 at 15 µM.

Experiment 3: Effect of low-dose CLA supplementation during maturation only or throughout the entire embryo production process.

Supplementation of CLA isomers at different points throughout the embryo production process did not affect cleavage rates among groups ($P > 0.05$; Table 6). However, blastocyst rates were depressed when CLA *t*10, *c*12 was supplemented both

during IVM and during the post-IVF culture period. CLA *c*9, *t*11 only affected blastocyst rate when provided at IVC. Blastomere counts (Figure 5) did not differ among groups treated with 15 μM of either CLA isomer ($P > 0.05$).

Experiment 4: Effect of CLA supplementation on embryo viability following vitrification and thawing.

Although there was some variation in embryo cleavage rates among groups, overall percentages were within an acceptable range (86 to 95%; Table 7). Analyses for blastocyst survival rates showed that treatment with 100 μM of CLA *c*9, *t*11 during IVC prior to vitrification performed the best among all the vitrified groups, but was not statistically different from control or CLA *t*10, *c*12 100 μM IVC. Treatment levels *t*10, *c*12 CLA 15 μM IVM and *c*9, *t*11 CLA 15 μM IVM yielded the lowest survival values (less than 20%; Table 7). Analyses of blastomere cell counts showed that embryos treated with 100 μM *c*9, *t*11 CLA prior to vitrification had the highest blastomere count among all the vitrified groups ($P < 0.01$; Figure 6) with cell count comparable ($P > 0.05$) to that in non-vitrified control embryos (Control+).

DISCUSSION

Previous reports have shown that CLA isomers, as other PUFAs, affect lipid configuration and membrane integrity along with cell signaling (Fouladi-Nashta et al., 2009; Marei et al., 2009; Al Darwich et al., 2010). Hence the beneficial effects of CLA during culture and on survival of embryos following cryopreservation have been explored (Pereira at al., 2007; Pereira et al., 2008). However, the effect of CLA isomers on bovine oocytes and embryos in the context of oocyte maturation and

embryo development to blastocysts requires further investigation (Lapa et al., 2011). The main objective of the current study was to systematically assess the effect of CLA supplementation at different stages during the in vitro embryo production process on maturation, cleavage and preimplantation blastocyst development.

In the first experiment, in which we performed parthenogenetic activation of oocytes, CLA supplementation during IVM had no effect on maturation, cleavage or blastocyst rates. However, when CLAs were supplemented at high doses during the entire culture period, we observed significant differences in maturation and cleavage rates which were generally negative and dose dependent. Subsequent trials with low CLA concentrations showed neither statistically significant differences nor benefits of supplementation on embryo development after parthenogenetic activation. Across this experiment, 15-100 μM CLA *t*10, *c*12 increased cleavage rates without concomitant increases in blastocyst rates, a result for which we do not have a reasonable explanation.

Previous studies have shown that PUFAs play a role in follicular cell steroidogenesis. Indeed, a study of the effect of PUFAs upon granulosa and theca cells in culture showed that fatty acid supplementation increased P4 production in theca cells (Hughes et al., 2011). Contradictory effects of progesterone on oocyte and embryo developmental competence have been reported (Fair and Lonegan, 2012). For example, one study indicated a detrimental effect of progesterone on in vitro matured oocytes (Fukui et al., 1982) contrasting with others reporting a beneficial effect of progesterone during embryo culture (Pereira et al., 2009; Fergusson et al., 2012). In the current study, addition of CLAs during IVM upregulated progesterone levels in a

dose dependent manner, which in turn was inversely correlated to embryo development. The fact that progesterone was upregulated by the effects of CLA upon cumulus cells (enclosing the oocytes), is interesting because this granulosa cell phenotype, to our knowledge, has not been reported. Given that the oocyte and cumulus cells are considered a complex, we suggest that CLAs exerted a global effect upon COCs. In this regard, one potential explanation may be that CLAs affect signaling transducers such as Sma and Mad-related (SMAD) intracellular signal transducers that are present in the cumulus cells thereby altering sterol synthesis (Gilchrist, 2011). Alternatively, CLAs may have affected the morphogenetic gradient generated by oocyte secreted factors that in turn maintain cumulus cell phenotype and other functions related to sterol synthesis (Gilchrist, 2011).

Given that high CLA doses exerted detrimental effects on parthenogenetic embryos in experiment one, we decided to test the effects of CLA supplementation upon IVF-produced embryos using low CLA supplemental doses. Results of the IVF experiments were overall consistent with those obtained with parthenotes. For instance, supplementation with low dose CLA isomers throughout the in vitro embryo production process yielded no significant differences in terms of cleavage rates and only minor differences in blastocyst rates. However, concentrations higher than 15 μM CLA resulted in a significant reduction in blastocyst rates, particularly when using the CLA *t*10, *c*12 isomer. Although we observed lower blastocyst rates for both CLA isomers when used during either IVM or IVC when compared to control, CLA *c*9, *t*11 at both concentrations tested yielded higher values than with CLA *t*10, *c*12. In this regard, a consistent observation throughout our study was that long term CLA

supplementation, especially with the higher doses, was detrimental to embryos. These findings are in agreement with Hughes et al. (2011) who found long term culture in the presence of PUFAs resulted in poorer quality embryos. In addition, PUFAs may affect the stearoyl-CoA desaturase (Scd) gene that in turn has direct effects on membrane fluidity and lipid metabolism (Keating et al., 2006). However, further experiments need to be performed to confirm this hypothesis.

Al Darwich et al. (2010) showed that relative gene expression of lipogenic transcription factors such as SREBP1 and Scd were numerically higher in embryos treated with 100 μM *t*10, *c*12. In this regard, results from the Nile red staining experiment were consistent with CLA *t*10, *c*12 yielding a higher epifluorescent signal for lipid content. Based upon the work of Granlund et al. (2005), we speculate that a glucose transporter could be affected by CLA *t*10, *c*12 thus exerting its effects on lipogenic transcription factors such as LXRα. Besides being involved in its own upregulation, LXRα is involved in the regulation of other lipogenic genes such as SREBP1 and FAS. It was obvious that embryos treated with ≥ 25 μM CLA *t*10,*c*12 appeared less viable quantitatively (i.e. reduced blastocyst and survival rates) and, more importantly, qualitatively (i.e. reduced blastomere cell counts). Alternatively, CLA *t*10, *c*12 may have redirected glucose toward the triglyceride synthesis pathway, thus compromising glycolysis, a pathway that is physiologically enhanced during morula compaction. If this is true, the redirection of glucose fate and the alteration in lipogenesis may have been detrimental for embryos, and further aggravated by lower metabolism of internal lipids due to the lack of L-carnitine in vitro (Sutton-McDowall et al., 2012).The relative transcript abundance of two anti-apoptotic markers whose

expression is related to embryo survival (*survivin and HSP 70.1*) was reduced at both CLA *t*10, *c*12 concentrations whereas supplementation with *c*9, *t*11 CLA seemed to maintain a similar relative expression to that in the control group. With this body of evidence we report that CLA *t*10, *c*12 was consistently detrimental not only upon blastocyst rates, but also upon the developmental performance as evidenced by qualitative markers.

Cytoplasmic lipid content of bovine oocytes/embryos and other species such as equine, swine and canine, is physiologically higher than that in the human and murine species (Sturmey et al., 2009). However, recent evidence indicates that high levels of fatty acids disturb mitochondrial activity during in vitro maturation (van Hoeck et al., 2013). In addition, excessive lipid accumulation may affect the developmental performance of embryos undergoing cryopreservation procedures. For that reason we tested the ability of embryos to survive cryopreservation following supplementation with either the low 15 μM CLA dose or under a higher 100 μM dose for a short time period as tested in previous studies (Pereira et al., 2007; Al Darwich et al., 2010). In our study, embryos obtained from IVM supplemented with 15 μM CLAs had poorer survival rates after thawing. However, short incubation with CLAs from the morula to early blastocyst stage, especially for isomer *c*9, *t*11 resulted in higher survival rates as compared to the control group that underwent vitrification. Furthermore, while our results appear to contradict those from previous studies (Pereira et al., 2007, 2008) regarding embryo development and performance after vitrification, 100 μM isomer *c*9,*t*11 supplemented for 36 hr prior to vitrification resulted in similar blastomere counts to our quality control group not undergoing vitrification. This finding supports

the notion that during the short incubation period, supplementation with this CLA isomer exerted beneficial effects through improved membrane fluidity (Zeron et al., 2002).

CONCLUSION

Through a systematic series of experiments, we studied the effect of CLA isomers during the in vitro embryo production process of parthenogenetically activated or in vitro fertilization embryos up to d-8 post activation or post fertilization stage, respectively. Overall, the effects of CLA on embryo development and performance were detrimental especially for isomer *t*10, *c*12 and for longer periods of culture. Embryos supplemented with CLA *c*9, *t*11 100 μM during a short incubation time (36 hr) showed the highest survival rates and blastomere cell counts. Additional benefits from CLAs supplementation, other than better performance after thawing vitrified embryos, require further study.

ACKNOWLEDGEMENTS

The authors would like to thank the Consejo Nacional de Ciencia y Tecnología (CONACyT, México) as well as the Department of Animal Sicence (Cornell University) for providing financial support to VAA for the PhD program. Many thanks to Cargill Wyalusing, PA for providing fresh bovine ovaries on a weekly basis. We also appreciate the kind support from Genex Cooperative Inc., Ithaca, NY for donating bovine semen for our IVF trials. We really appreciate Dr. Cohen's lab for providing access to and advice for epifluorescence microscopy. Special thanks to Professors

D.E. Bauman and V. Selvaraj (Cornell University, Animal Science Department) for their insightful thoughts during the development of this manuscript.

REFERENCES

Al Darwich, A., C. Perreau, M. H. Petit, P. Papillier, J. Dupont, D. Guillaume, P. Mermillod and F. Guignot. 2010. Effect of PUFA on embryo cryoresistance, gene expression and AMPKalpha phosphorylation in IVF-derived bovine embryos. Prostaglandins Other Lipid Mediat. 93:30-36.

Bastos, G. M., P. B. Goncalves and V. Bordignon. 2008. Immunolocalization of the high-mobility group N2 protein and acetylated histone H3K14 in early developing parthenogenetic bovine embryos derived from oocytes of high and low developmental competence. Mol. Reprod. Dev. 75:282-290.

Bauman, D. E., J. W. Perfield 2nd, K. J. Harvatine and L. H. Baumgard. 2008. Regulation of fat synthesis by conjugated linoleic acid: Lactation and the ruminant model. J. Nutr. 138:403-409.

Bonilla, L. and P. J. Hansen. 2009. Determining lipid content in embryos using Nile red fluorescence. Date of access (Feb-2010) http://www.animal.ufl.edu/hansen/Protocols/Detection%20of%20lipids%20in%20embryos%20by%20Nile%20Red%20fluorescence.pdf

Bougnoux, P., N. Hajjaji, K. Maheo, C. Couet and S. Chevalier. 2010. Fatty acids and breast cancer: Sensitization to treatments and prevention of metastatic re-growth. Prog. Lipid Res. 49:76-86.

Elrod, C. C. and W. R. Butler. 1993. Reduction of fertility and alteration of uterine pH in heifers fed excess ruminally degradable protein. Journal of Animal Science. 71:694-701.

Fair, T. and P. Lonergan. 2012. The role of progesterone in oocyte acquisition of developmental competence. Reproduction in Domestic Animals. 47:142-147.

Fouladi-Nashta, A. A., K. E. Wonnacott, C. G. Gutierrez, J. G. Gong, K. D. Sinclair, P. C. Garnsworthy and R. Webb. 2009. Oocyte quality in lactating dairy cows fed on high levels of n-3 and n-6 fatty acids. Reproduction. 138:771-781.

Gilchrist, R. B. 2011. Recent insights into oocyte-follicle cell interactions provide opportunities for the development of new approaches to in vitro maturation. Reprod. Fertil. Dev. 23:23-31.

Goedecke, J. H., D. E. Rae, C. M. Smuts, E. V. Lambert and M. O'Shea. 2009. Conjugated linoleic acid isomers, t10c12 and c9t11, are differentially incorporated into adipose tissue and skeletal muscle in humans. Lipids. 44:983-988.

Granlund, L., J. I. Pedersen and H. I. Nebb. 2005. Impaired lipid accumulation by trans10, cis12 CLA during adipocyte differentiation is dependent on timing and length of treatment. Biochim. Biophys. Acta. 1687:11-22.

Holm, P., P. J. Booth, M. H. Schmidt, T. Greve and H. Callesen. 1999. High bovine blastocyst development in a static in vitro production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology. 52:683-700.

Hughes, J., W. Y. Kwong, D. Li, A. M. Salter, R. G. Lea and K. D. Sinclair. 2011. Effects of omega-3 and -6 polyunsaturated fatty acids on ovine follicular cell steroidogenesis, embryo development and molecular markers of fatty acid metabolism. Reproduction. 141:105-118.

Janovick-Guretzky, N. A., H. M. Dann, D. B. Carlson, M. R. Murphy, J. J. Loor and J. K. Drackley. 2007. Housekeeping gene expression in bovine liver is affected by physiological state, feed intake, and dietary treatment. J. Dairy Sci. 90:2246-2252.

Keating, A. F., J. J. Kennelly and F. Q. Zhao. 2006. Characterization and regulation of the bovine stearoyl-CoA desaturase gene promoter. Biochem. Biophys. Res. Commun. 344:233-240.

Lapa, M., C. Marques, S. Alves, M. Vasques, M. Baptista, I. Carvalhais, M. Silva Pereira, A. Horta, R. Bessa and R. Pereira. 2011. Effect of trans-10 cis-12 conjugated linoleic acid on bovine oocyte competence and fatty acid composition. Reproduction in Domestic Animals. 46:904-910.

Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods. 25:402-408.

Lock, A. L. and D. E. Bauman. 2004. Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. Lipids. 39:1197-1206.

Malcuit, C., M. Kurokawa and R. A. Fissore. 2006. Calcium oscillations and mammalian egg activation. J. Cell. Physiol. 206:565-573.

Marei, W. F., D. C. Wathes and A. A. Fouladi-Nashta. 2009. The effect of linolenic acid on bovine oocyte maturation and development. Biol. Reprod. 81:1064-1072.

Parrish, J. J., J. Susko-Parrish, M. A. Winer and N. L. First. 1988. Capacitation of bovine sperm by heparin. Biol. Reprod. 38:1171-1180.

Parrish JJ, Susko-Parrish JL, First NL, 1989: Capacitation of bovine sperm by heparin: inhibitory effect of glucose and role of intracellular pH. Biol Reprod 41, 683–699.

Pereira, R. M., M. C. Baptista, M. I. Vasques, A. E. Horta, P. V. Portugal, R. J. Bessa, J. C. Silva, M. S. Pereira and C. C. Marques. 2007. Cryosurvival of bovine blastocysts is enhanced by culture with trans-10 cis-12 conjugated linoleic acid (10t,12c CLA). Anim. Reprod. Sci. 98:293-301.

Pereira, R. M., I. Carvalhais, J. Pimenta, M. C. Baptista, M. I. Vasques, A. E. Horta, I. C. Santos, M. R. Marques, A. Reis, M. S. Pereira and C. C. Marques. 2008. Biopsied and vitrified bovine embryos viability is improved by trans10, cis12 conjugated linoleic acid supplementation during in vitro embryo culture. Anim. Reprod. Sci. 106:322-332.

Pereira, R. M. and C. C. Marques. 2008. Animal oocyte and embryo cryopreservation. Cell. Tissue Bank. 9:267-277.

Pers-Kamczyc, E., E. Warzych, J. Peippo and D. Lechniak. 2010. Growth hormone exerts no effect on the timing of the first zygotic cleavage in cattle. Theriogenology. 74:581-595.

Rief, S., F. Sinowatz, M. Stojkovic, R. Einspanier, E. Wolf and K. Prelle. 2002. Effects of a novel co-culture system on development, metabolism and gene expression of bovine embryos produced in vitro. Reproduction. 124:543-556.

Rizos, D., A. Gutierrez-Adan, P. Moreira, C. O'Meara, T. Fair, A. C. Evans, M. P. Boland and P. Lonergan. 2004. Species-related differences in blastocyst quality are associated with differences in relative mRNA transcription. Mol. Reprod. Dev. 69:381-386.

Robinson, R., P. Pushpakumara, Z. Cheng, A. Peters, D. Abayasekara and D. Wathes. 2002. Effects of dietary polyunsaturated fatty acids on ovarian and uterine function in lactating dairy cows. Reproduction. 124:119-131.

Sampath, H. and J. M. Ntambi. 2005. Polyunsaturated fatty acid regulation of genes of lipid metabolism. Annu. Rev. Nutr. 25:317-340.

Staples, C. R., J. M. Burke and W. W. Thatcher. 1998. Influence of supplemental fats on reproductive tissues and performance of lactating cows. J. Dairy Sci. 81:856-871.

Susko-Parrish, J. L., M. L. Leibfried-Rutledge, D. L. Northey, V. Schutzkus and N. L. First. 1994. Inhibition of protein kinases after an induced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion. Dev. Biol. 166:729-739.

Sutton-McDowall, M. L., D. Feil, R. L. Robker, J. G. Thompson and K. R. Dunning. 2012. Utilization of endogenous fatty acid stores for energy production in bovine preimplantation embryos. Theriogenology. 77:1632-1641.

Sturmey, R., A. Reis, H. Leese and T. McEvoy. 2009. Role of fatty acids in energy provision during oocyte maturation and early embryo development. Reproduction in Domestic Animals. 44:50-58.

Zeron, Y., D. Sklan and A. Arav. 2002. Effect of polyunsaturated fatty acid supplementation on biophysical parameters and chilling sensitivity of ewe oocytes. Mol. Reprod. Dev. 61:271-278.

TABLES

Table 1. Primers used for embryo RNA transcript quantification.

Name	Primer sequence (5' -> 3')	Fragment size (bp)	Temp. (^{o}C)	Reference
RPS9 Forward	CCTCGACCAAGAGCTGAAG	62	60	Janovick-Guretzky et al. (2007)
RPS9 Reverse	CCTCCAGACCTCACGTTTGTTC			
Survivin Forward	CATTCATCCGGTTGTGCTTTCC	175	55	Rizos et al. (2004)
Survivin Reverse	GCTGCTCAATGGCACAGCGGAC			
Hsp 70.1 Forward	CCCCAAGCTATGTCGCCTT	239	60	Rief et al. (2002)
Hsp 70.1 Reverse	GGATTCATTGCGACTTGGTT			

Table 2. Effects of high dose CLA supplementation in maturation medium upon parthenogenetically activated embryo development (Experiment 1).

Group	Total oocytes*	Maturation rate*(%)	Cleavage rate*(%)	Blastocyst rate*[1] (%)
Control	35.0 ± 3.0	94.0 ± 2.0	83.7 ± 2.5	31.5 ± 6.2
*t*10 *c*12 50 μM	35.7 ± 2.7	90.9 ± 5.1	81.6 ± 6.2	25.2 ± 8.1
*t*10 *c*12 100 μM	35.3 ± 0.3	90.5 ± 2.6	80.2 ± 6.6	29.5 ± 1.3
*t*10 *c*12 200 μM	35.3 ± 2.0	88.9 ± 2.5	76.3 ± 7.7	33.4 ± 10.5
*c*9 *t*11 50 μM	34.7 ± 1.9	93.3 ± 0.7	85.4 ± 4.3	22.9 ± 4.7
*c*9 *t*11 100 μM	35.0 ± 1.0	94.2 ± 2.9	86.3 ± 6.8	28.5 ± 7.0
c9 t11 200 μM	35.0 ± 0.6	89.6 ± 1.7	78.3 ± 3.5	31.1 ± 9.1

*mean ± SEM from 3 replicates. Means within columns were not significantly different.
[1]blastocyst rates were based on percentages of the original oocyte number.

Table 3. Effects of high dose CLA supplementation during the entire culture period (pre- and post-activation) upon parthenogenetically activated embryo development (Experiment 1).

Group	Total oocytes*	Maturation rate*(%)	Cleavage rate*(%)	Blastocyst rate*1(%)
Control	35.7 ± 1.1	94.4 ± 1.5^{abc}	76.2 ± 2.6^{bc}	28.0 ± 4.2^{a}
*t*10 *c*12 50 μM	35.7 ± 1.1	97.1 ± 1.1^{a}	87.2 ± 2.8^{a}	17.9 ± 1.8^{bc}
*t*10 *c*12 100 μM	36.2 ± 1.1	95.4 ± 1.0^{ab}	80.9 ± 2.2^{ab}	11.5 ± 2.1^{cd}
*t*10 *c*12 200 μM	37.0 ± 2.5	90.1 ± 1.5^{d}	76.1 ± 3.4^{bc}	07.8 ± 0.9^{d}
*c*9 *t*11 50 μM	35.8 ± 1.4	91.2 ± 1.6^{cd}	71.5 ± 3.9^{c}	23.3 ± 2.2^{ab}
*c*9 *t*11 100 μM	35.3 ± 1.6	91.3 ± 1.6^{cd}	77.1 ± 3.4^{bc}	16.4 ± 1.5^{c}
c9 t11 200 μM	36.7 ± 1.5	92.2 ± 1.2^{bcd}	80.6 ± 3.8^{abc}	15.2 ± 1.2^{c}

*mean ± SEM from 6 replicates. Means within a column not sharing the same letter are significantly different ($P < 0.05$).
[1]blastocyst rates were based on percentages of the original oocyte number.

Table 4. Effects of low dose CLA supplementation during the entire culture period (pre- and post-activation) on parthenogenetically activated embryo development (Experiment 1).

Group	Total oocytes*	Maturation rate*(%)	Cleavage rate*(%)	Blastocyst rate*1(%)
Control	34.2 ± 1.0	89.0 ± 1.2	67.7 ± 2.8^{cd}	20.7 ± 1.9
*t*10 *c*12 15 μM	35.2 ± 1.6	94.5 ± 1.8	86.6 ± 2.0^{a}	19.4 ± 1.0
*t*10 *c*12 25 μM	33.8 ± 0.6	93.0 ± 2.4	75.5 ± 3.2^{abcd}	18.7 ± 4.7
*t*10 *c*12 50 μM	34.6 ± 1.4	91.4 ± 2.9	78.8 ± 6.1^{abc}	14.2 ± 1.0
*c*9 *t*11 15 μM	33.6 ± 1.4	92.7 ± 2.4	79.2 ± 4.1^{ab}	17.9 ± 3.2
*c*9 *t*11 25 μM	35.0 ± 1.1	92.0 ± 1.6	69.9 ± 3.8^{bcd}	18.7 ± 1.9
c9 t11 50 μM	34.8 ± 2.0	91.1 ± 1.5	64.3 ± 3.7^{d}	19.3 ± 2.1

Means within a column not sharing the same letter are significantly different ($P < 0.05$).
*mean ± SEM from 5 replicates.
[1]blastocyst rates were based on percentages of the original oocyte number.

Table 5. Effects of low dose CLA supplementation throughout culture on production of embryos by in vitro fertilization (Experiment 2).

Group	Total oocytes[*]	Cleavage rate[*](%)	Blastocyst rate[*1] (%)
Control	40.5 ± 1.6	86.9 ± 1.5	38.2 ± 2.3[a]
*t*10 *c*12 15 μM	42.5 ± 1.8	89.6 ± 1.8	36.6 ± 4.8[a]
*t*10 *c*12 25 μM	42.8 ± 1.7	82.4 ± 3.0	25.5 ± 2.1[b]
*c*9 *t*11 15 μM	42.3 ± 2.0	83.8 ± 1.9	38.2 ± 2.3[a]
*c*9 *t*11 25 μM	41.8 ± 1.5	82.1 ± 3.9	32.9 ± 2.4[ab]

[*]mean ± SEM from 4 replicates. Means within a column not sharing the same letter are significantly different ($P < 0.05$).
[1]blastocyst rates were based on percentages of the original oocyte number.

Table 6. Effects of low dose CLA supplementation during in vitro maturation (IVM) or throughout the entire in vitro embryo production (IVC) period (Experiment 3).

Group	Total oocytes[*]	Cleavage rate[*](%)	Blastocyst rate[*1](%)
Control	45.3 ± 1.9	90.1 ± 0.2	34.18 ± 1.2[a]
*t*10 *c*12 15 μM IVM	44.8 ± 2.8	92.3 ± 1.0	28.61 ± 1.2[b]
*t*10 *c*12 15 μM IVC	44.5 ± 2.6	87.1 ± 3.2	20.55 ± 2.4[c]
*c*9 *t*11 15 μM IVM	45.0 ± 2.3	88.4 ± 2.4	33.97 ± 1.6[a]
*c*9 *t*11 15 μM IVC	46.0 ± 3.0	90.2 ± 1.1	27.65 ± 1.2[b]

[*]mean ± SEM from 4 replicates. Means within a column not sharing the same letter are significantly different ($P < 0.05$).
[1]blastocyst rates were based on percentages of the original oocyte number.

Table 7. The effect of CLA supplementation during in vitro maturation of oocytes (IVM) or embryo culture (IVC) on embryo viability following vitrification (Experiment 4).

Group	Total oocytes*	Cleavage rate*(%)	Embryos (n)*	Survival rate*[1](%)
Control	42.0 ± 0.0	90.5 ± 2.3[ab]	30.5 ± 0.7	39.24 ± 4.7[ab]
Control +[2]	43.5 ± 0.5	95.4 ± 2.2[a]	NA	NA
*t*10 *c*12 15 μM IVM[3]	42.5 ± 0.5	92.9 ± 0.1[a]	30.5 ± 2.1	05.26 ± 1.4[c]
*t*10 *c*12 100 μM IVC[4]	40.0 ± 2.0	86.3 ± 0.5[b]	31.5 ± 0.7	30.41 ± 1.8[ab]
*c*9 *t*11 15 μM IVM[3]	42.5 ± 0.5	90.6 ± 0.1[ab]	32.5 ± 0.7	21.63 ± 9.1[bc]
*c*9 *t*11 100 μM IVC[4]	40.5 ± 0.5	86.4 ± 1.4[b]	27.5 ± 3.5	44.36 ± 5.6[a]

*mean ± SEM from 2 replicates. Means within a column not sharing the same letter are significantly different ($P < 0.05$).

[1]Survival rates were based on the numbers of vitrified embryos.

[2]Group not undergoing vitrification, blastocysts were used for blastomere cell counts.

[3]CLAs supplemented only at IVM. The remainder of the culture period was performed with CLA-free medium.

[4]CLAs supplemented on d-5 post fertilization embryos for a period of 36 hours.

FIGURES

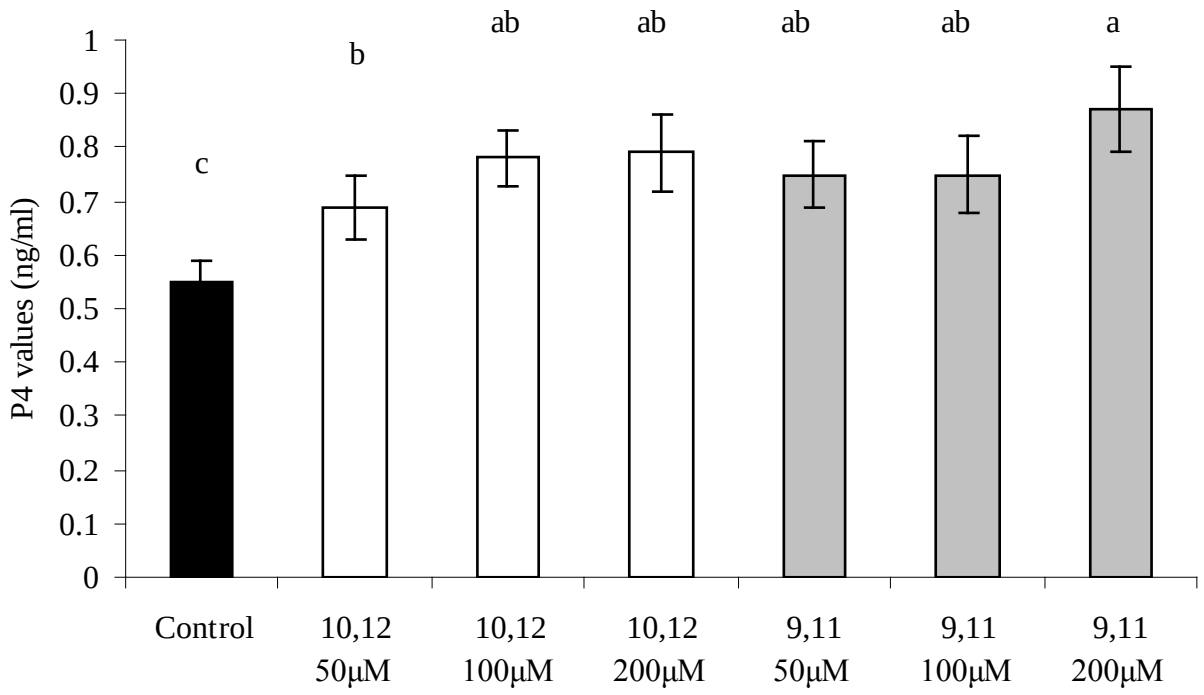

Figure 1. Effect of high doses of CLA isomers during oocyte maturation on progesterone concentrations in maturation medium (Experiment 1).

Means ± SEM not sharing the same letter are significantly different ($P < 0.05$).

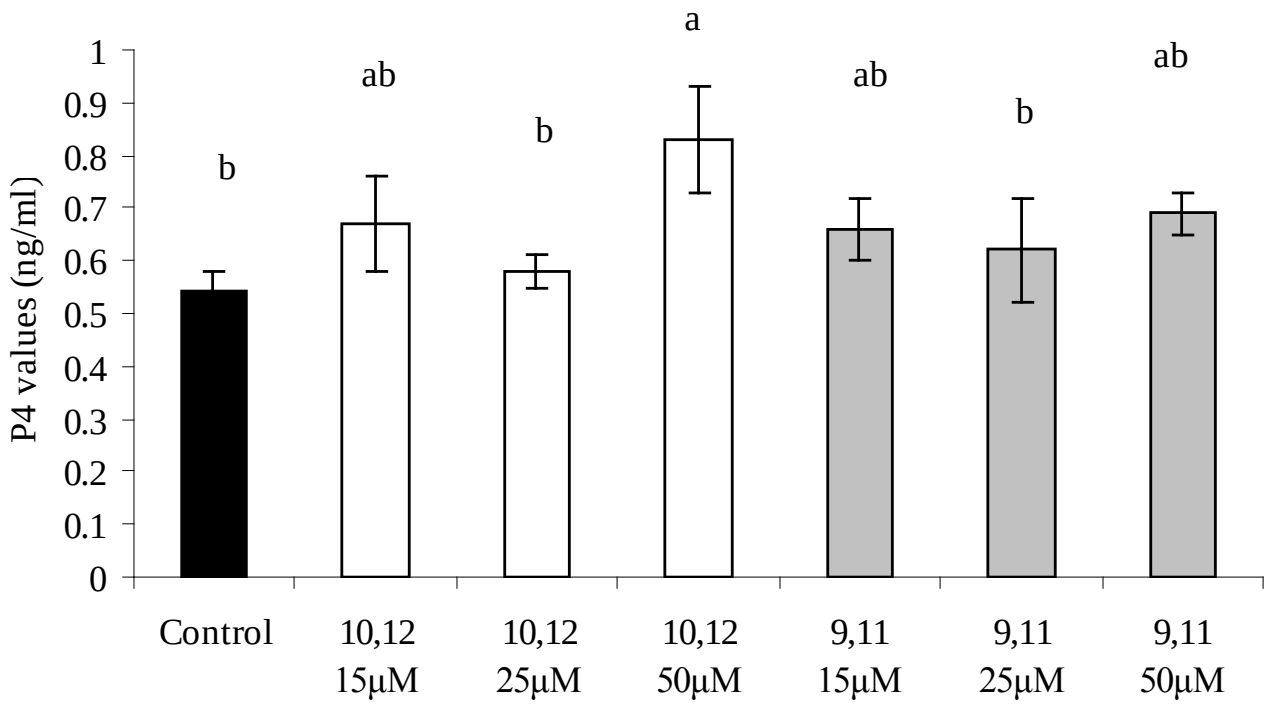

Figure 2. Effect of low dose CLA-isomer supplementation during oocyte maturation upon progesterone concentrations in the medium (Experiment 1).
Means ± SEM not sharing the same letter are significantly different ($P < 0.05$).

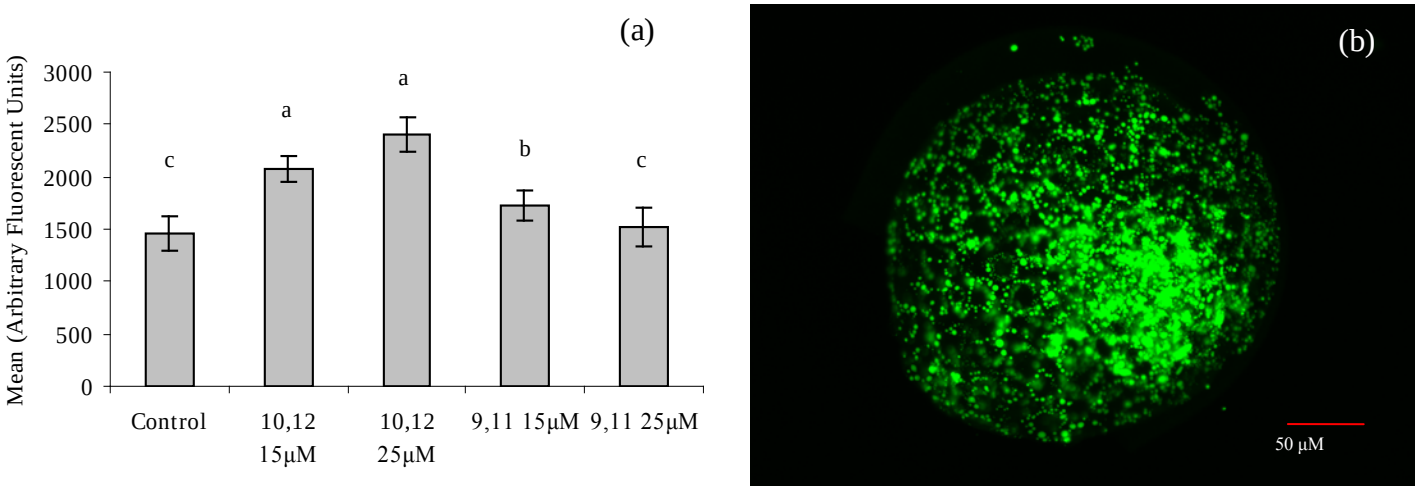

Figure 3. Effect of CLA supplementation on lipid content in d-8 IVF embryos.
(a) Nile red staining technique (Bonilla and Hansen 2009) was used to measure differences among groups. Means not sharing the same letter are significantly different ($P < 0.05$). Each error bar represents 1 standard error of the mean. (b) Representative embryo stained with Nile red at d-8 post insemination.

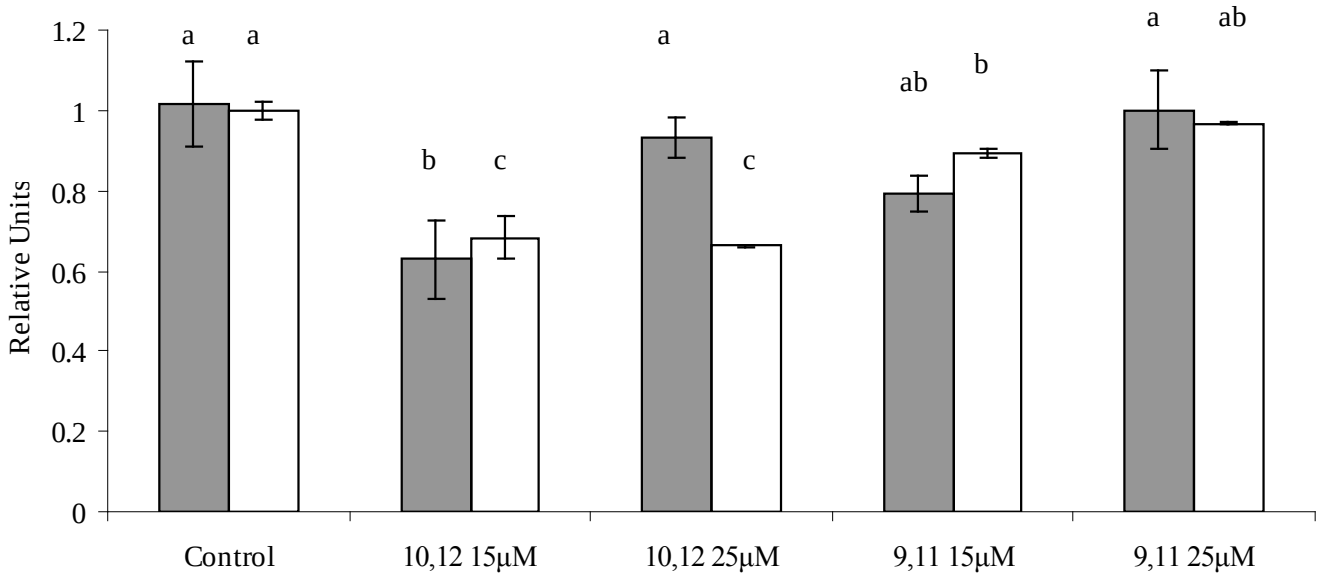

Figure 4. Effect of CLA supplementation on gene expression in d-8 bovine IVF embryos. HSP 70.1 (solid bars) and Survivin (open bars). Values represent means ± SEM (n=13 ± 4 embryos per group per replicate) with statistical differences ($P < 0.05$) from control indicated by different superscripts.

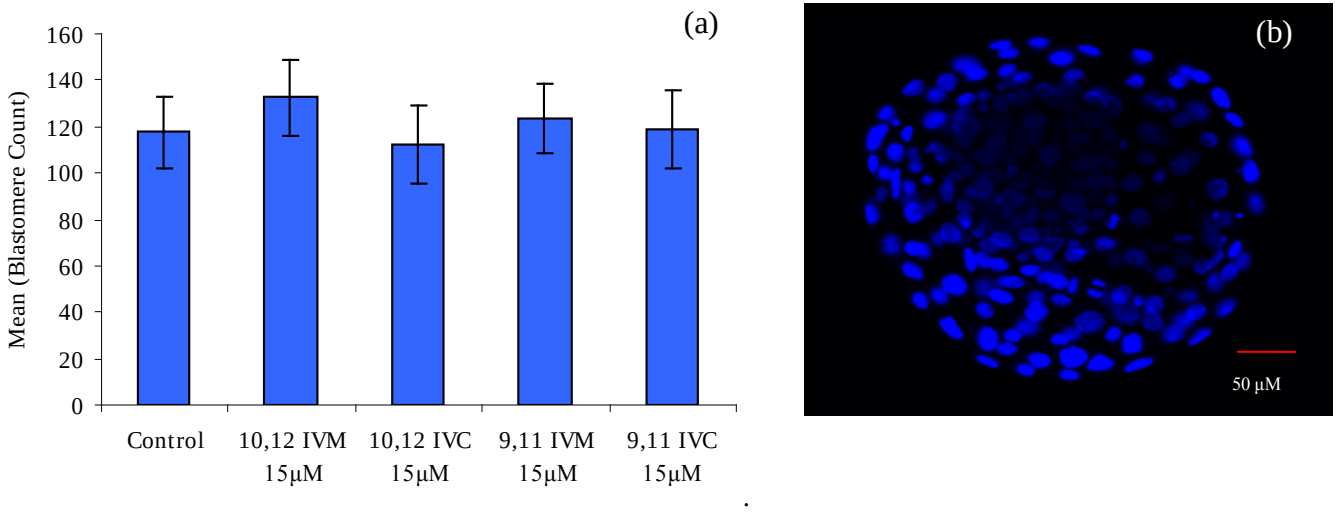

Figure 5. Effect of CLA supplementation (15 µM) during in vitro oocyte (IVM) or throughout culture (IVC) on blastomere counts at d-8 post fertilization.

Blastocyst cell numbers (a) are presented as mean ± SEM. No statistical differences ($P > 0.05$) were detected. (b) Blastomere count was determined by staining the embryos with Hoechst 33342 (n = 66 embryos) and counting under an epifluorescent microscope at 40X.

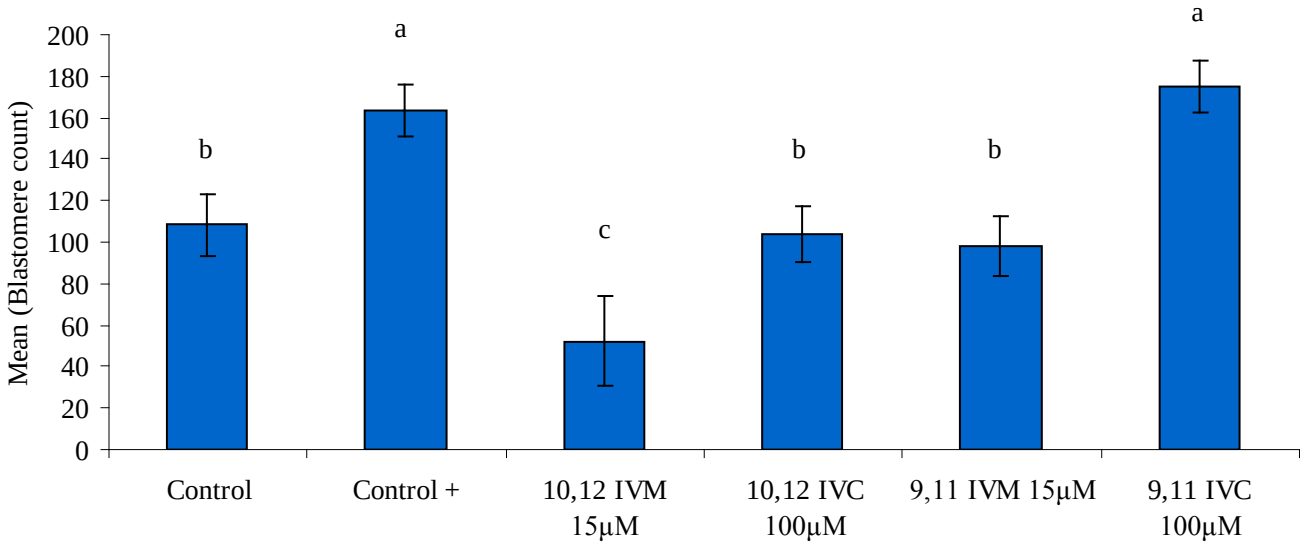

Figure 6. Effect of CLA supplementation at different time points.
15 µM at in vitro maturation or 100 µM at in vitro embryo culture were supplied during the in vitro preimplantation embryo production for mean blastomere counts at post survival assessment (equivalent to d-8 post fertilization. Mean ± SEM blastocyst cell numbers not sharing the same letter are significantly different ($P < 0.05$). Blastomere count was determined by staining the embryos (n = 80) with Hoechst 33342. Control + embryos did not undergo vitrification.

CHAPTER FOUR: ENHANCING GLUCOSE METABOLISM OF BOVINE EMBRYOS *IN VITRO* IN PREPARATION FOR THE HYPOOXYGENATED UTERINE ENVIRONMENT.

V. A. Absalón-Medina[†], S. H. Cheong[‡], R. O. Gilbert[‡], and W. R. Butler[†*]

In final preparation for submission to Theriogenology

[†]College of Agriculture and Life Sciences, Animal Science Department. Cornell University, Ithaca, NY 14853. USA.

[‡]College of Veterinary Medicine, Department of Clinical Sciences. Cornell University, Ithaca, NY 14853. USA

[*]Correspondence e-mail: wrb2@cornell.edu

ABSTRACT

Metabolic regulators (MR) such as 2, 4—dinitrophenol (DNP) and phenazine ethosulfate (PES) can improve glucose metabolism of bovine embryos in vitro via two different pathways viz. glycolysis and pentose phosphate pathways. These metabolic enhancers are intended to stimulate the metabolic switch from low to high glucose utilization at the morula stage. This metabolic adjustment occurs naturally along the oviduct during migration of the preimplantation embryo toward the hypooxygenated environment of the uterus. The objectives of this study were to evaluate the effects of these MR, individually or in combination, on development of bovine embryos obtained by in vitro fertilization procedures. Further, the effects of MR in combination with a conjugated linoleic acid isomer (CLA—*cis*9, *trans*11) were evaluated on viability of embryos after vitrification. In Experiment 1, embryos were supplemented with PES (0.3 μM), DNP (10 μM), the combination and Control. In Experiment 2, embryos were supplemented with lower PES (0.15 μM) in combination with DNP at 3 doses (5, 10 and 30 μM). Two quality control groups were included: one group remained untreated and the other was the combined MR treatment from Experiment 1 (PES 0.3 μM + DNP 10 μM). Experiment 3 consisted of 4 treatment groups. The best treatment from Experiments 1 and 2 (DNP 10 μM, PES 0.3 μM) was supplemented with 0, 50, or 100 μM CLA *cis*9, *trans*11 on day 7 for the subsequent 36 hours before vitrification. In addition, one control group remained MR and CLA-free. In Experiment 1 and 2, blastocyst rates significantly increased from using the combination of MR (DNP 10 μM, PES 0.3 μM; control +) as compared to control and other treatment combinations (> 40% vs. ~30%; $P < 0.05$). The best MR combination

resulted in expanded-stage blastocyst rates higher than control (28% vs 15%; $P < 0.05$). MR resulted in a marked dose-dependent effect on the triglyceride content of expanded stage embryos ($P < 0.05$) and had a positive effect in blastomere number of expanded embryos ($P < 0.05$). Treatment with PES alone negatively affected average blastomere numbers (126) when compared to other groups (DNP = 148; combination = 142) including control (135). In Experiment 3, MR treated embryos resulted in higher re-expansion rates after embryos were thawed when compared to control (71% vs. 60%, respectively). In addition, CLA treated embryos showed the lowest re-expansion rates and this effect was more evident with the highest CLA dose (CLA 50 μM = 51%, CLA 100 μM =32%). Among all treatment groups, vitrified embryos showed no differences in terms of cytoskeleton integrity post-thaw. In conclusion, addition of MR improved blastocyst rates, but most importantly expanded stage embryo rates were about 2-fold higher when compared to control. Following MR alone, embryos showed high capability to withstand the stressful procedures of vitrification and were similar to vitrified control group. Treatment with CLA did not provide any further additive effect on blastocyst rates nor on benefits after vitrification.

INTRODUCTION

Soon after fertilization *in vivo*, the developing embryo is exposed to a dynamic environment as its metabolism changes from early to late preimplantation stages. Pyruvate and lactate are the preferred energy substrates during precompaction stages, coincident with location in the relatively well oxygenated environment of the oviduct. As the embryo reaches the uterus, usually at compacted morula or early blastocyst stages, the local oxygen tension decreases and glucose metabolism is enhanced to accommodate the higher demands for energy (Thompson et al., 2000). This metabolic switch from low to high glucose consumption would likely seem of equal importance in vitro to ensure an optimal environment for development prior to transfer of embryos to recipients.

One of the problems researchers faced earlier was the effect of glucose in media formulation at precompaction stages and the undesirable early onset of glycolysis resulted in embryo growth retardation or embryo development block (reviewed by Thompson, 2000; Ménézo et al., 2013). The adverse effect is reported to occur at the level of the rate limiting hexokinase that is alosterically regulated by substrate preference of the aforementioned tricarboxylic acids (Gardner et al., 2013). However, the discovery of supplementing the culture media with ethylenediaminetetraacetic acid (EDTA) overcame the embryo development block through inhibition of the so called “Crabtree-effect” and consequently, embryos were able to continue further cell cycles (Thompson et al., 2000). After the problem of early onset of glycolysis was solved by the addition of EDTA, it was acknowledged that

glucose metabolism needed to be enhanced at postcompaction stages. The use of chemicals with the opposite effect of EDTA has been demonstrated not only to enhance glucose consumption, but also to improve embryonic development and competence. On the one hand, the use of oxidative phosphorylation uncouplers such as 2,4—dinitrophenol (DNP) has been demonstrated to partially inhibit oxidative phosphorylation while enhancing glycolysis (Thompson et al., 2000). Although the exact mechanism of DNP remains to be elucidated, those authors proposed that partial inhibition of mitochondrial oxidative phosphorylation regulates the relative contribution of glycolytic ATP and overall favors glucose metabolism. On the other hand, phenazine ethosulfate (PES) acts as an electron acceptor for reduced nicotinamide adenine dinucleotide phosphate (NADPH) during oxidization to NADP. Thereby, PES enhances the pentose phosphate pathway and the conversion of glucose-6-phosphate to 6-phosphogluconate (De La Torre Sanchez et al., 2006).

Excessive lipid droplet formation is widely acknowledged to occur in embryos produced in vitro. There is a current interest in defining protocols that would reduce excessive cytoplasmic lipid droplet formation in this type of embryos, especially for those undergoing cryopreservation procedures. Polyunsaturated fatty acids, including conjugated linoleic acids isomers (CLA), can alter lipid membrane configuration and provide protection during cryopreservation procedures (Pereira et al., 2008; Marei et al., 2009). Our previous work indicated that inclusion of CLA (100 μM *cis*9, *trans*11) before vitrification improved post-thaw survival and embryo development (Absalón-Medina et al., 2011; Chapter Three). However, CLA was added from morula to early blastocyst stages, i.e. from d 5 to d 6.5 post IVF. For practical purposes we decided to

work with later stage embryos (7.5 d-post IVF), the embryonic stage routinely used for IVF embryo transfers. Furthermore, it has been reported that addition of PES to embryo culture media inhibits fatty acid synthesis in embryos (Sudano et al., 2011) and provided an important reason for testing the effects of PES in combination with DNP and CLA prior to vitrification procedures.

Since DNP and PES both stimulate the metabolism of glucose, but via two different metabolic pathways, the objectives of this study were to evaluate the effect of both chemicals, individually and in combination, on the rate of bovine embryo development. In addition, we evaluated the effects of these metabolic regulators in combination with CLA *cis*9, *trans*11 to observe the effects on triglyceride content and post thaw survival rates of vitrified embryos.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents were obtained from Sigma-Aldrich Co. (Saint Louis, MO, USA), unless otherwise indicated.

Experimental design

The effects of PES and DNP supplementation on bovine embryos.

In 8 replications, the media of bovine embryos was supplemented with PES (0.3 μM), DNP (10 μM) and the combination. Metabolic regulators were supplied from d 5 to d 8 post insemination. Embryos were stained and examined under epifluorescent microscopy for total embryo cell count and triglyceride content to assess embryo development and quality. For this we used only embryos at a homogenous developmental stage, i.e. expanded embryos (d-8 post insemination). A total of 1,249 COCs were used for this experiment.

The effects of the combination of low PES and varied concentrations of DNP on bovine embryos.

In 6 replications, bovine embryo media was supplemented with PES (0.15 μM) in combination with DNP at three levels (5, 10 and 30 μM). In addition, two quality control groups were included: One group remained untreated (negative control) and the other was the combination of PES 0.3 μM and DNP 10 μM from Experiment 1 (positive control). Metabolic regulators were supplied from d 5 to d 8 post insemination. Differential staining under epifluorescent microscopy was used to

determine total embryo cell count and triglyceride content as in experiment 1. A total of 1,247 COCs were used in this experiment.

The effects of metabolic regulators in combination with CLA *cis*9, *trans*11 on post-thaw viability of vitrified embryos.

This experiment consisted of the best treatment from the previous trials (PES [0.3 μM] and DNP [10 μM)]) in combination with 0, 50, or 100 μM CLA *cis*9, *trans*11 (4 treatment groups with three replicates). The Control group remained free of the metabolic regulators and CLA. The CLA was added at d-6 post fertilization for 36 hrs. Embryos were vitrified using a commercial vitrification kit (BoviPRO Vit Kit™ from Minitube) at d-7.5 post insemination (only expanded blastocyst stage). We measured post-thaw blastocyst re-expansion rates, total blastomere count and cytoskeleton integrity (*vide* staining procedures). A total of 620 COCs were used for this experiment.

Oocyte recovery and selection

Bovine ovaries were collected at a nearby abattoir (120 km) and transported to the laboratory in prewarmed lactated Ringer's solution at 30-35 °C. Cumulus oocyte-complexes (COCs) from 2-8 mm follicles were aspirated with an 18G hypodermic needle attached to an aspiration pump unit adjusted to a flow of 22.5 ± 2.5 ml of H_2O per minute. Follicular fluid supernatant was removed and the pellet containing COCs was transferred to a 15 ml tube where it was resuspended with holding media and the contents were poured gently into a 100 mm Petri dish. Holding media consisted of TCM-199 Hank's salts (Invitrogen, Grand Island, NY), 10% Fetal Calf Serum (FCS;

Invitrogen), 25 μg/ml of gentamicin, 0.2 mM Na-pyruvate, and heparin 5 μg/ml with a mOsm = 300 ± 1. Selection of COCs was based on morphological assessment as having several layers of cumulus granulosa cells and oocytes with a homogenous cytoplasm. The entire process was performed within 5 hr including transportation.

In vitro pre-maturation

Following the method of Albuz et al. (2010), selected COCs were pre-matured in holding media (*vide supra*) supplemented with adenylate cyclase activator, forskolin, and IBMX, a non-specific phosphodiesterase (PDE) inhibitor, at a final concentration of 100 μM and 500 μM, respectively, in order to increase overall cAMP levels and to inhibit overall PDEs in both cumulus cells and oocytes. Millimolar stock concentrations of the chemicals were stored at -20 °C dissolved in dimethylsulphoxide (DMSO) and used fresh in each trial. In groups of about 40 COCs, pre-maturation treatment was carried out under atmospheric and humidified conditions at 38.5 °C for 2 hr.

In vitro Maturation

Selected COCs were matured in groups of 40 ± 5 for 30 hours in 400 μL of TCM-199 (Earle's Salts) enriched with 10% FCS, 0.2 mM sodium pyruvate, 1 mM alanyl-glutamine, 0.1 mM taurine, 0.1 mM cysteamine, 1 μg/ml estradiol, 85 mU/ml bovine follicle stimulating hormone (FSH, SIOUX Biochemical, Inc., Sioux Center, IA), 20 μg/ml gentamicin, at pH 7.35 ± 0.02 and mOsm 300 ± 2 and covered with light mineral oil in a humidified atmosphere at 38.5 °C with 5% CO_2 in air. In addition, based on Albuz et al. (2010), COCs were cultured during the entire

maturation process with a type 3-specific PDE inhibitor, cilostamide (20 μM; Biomol, Plymouth Meeting, PA, USA).

In vitro fertilization

After a total of 30 hours, presumptive matured oocytes were transferred to a modified IVF medium (Fert-TALP; Parrish et al., 1988) supplemented with 0.5 mM fructose, 0.2 mM non-essential amino acids, 6 mg/ml BSA FFA Fraction V, 30 μM penicillamine, 15 μM hypotaurine, 1.5 μM epinephrine (PHE), 22 μg/ml heparin, 20 μg/ml gentamicin, covered with light mineral oil in a humidified atmosphere at 38.5 °C with 5% CO_2 in air for 18 hr (pH of 7.38 ± 0.01, mOsm 285 ± 1). Frozen semen straws (Genex, Ithaca NY, USA) from a single bull and from the same ejaculate were thawed at 37 °C for 30 sec. Motile sperm were separated from cryoprotectant, non-motile sperm, and debris by Percoll® double density gradient (90% and 45%) centrifugation at 300 x g for 20 min. Subsequently, sperm were washed twice in 5 ml of TL-Semen (pH 7.39 ± 0.01, mOsm 295 ± 2), which is a modified Tyrode's from Parrish et al. (1989) and centrifuged at 300 x g for 5 min to remove Percoll. Finally, sperm were adjusted to a final concentration of 1.5 x 10^6 sperm/ml using Fert-TALP media and applied to the oocytes.

In vitro embryo culture

A modified synthetic oviductal fluid (SOF) sequential medium was used (20 μg/ml gentamicin, pH 7.4 ± 0.01, mOsm 275 ± 5, humidified atmosphere at 38.5 °C with 5% CO_2, 7% O_2, and 88% N_2 [Holm et al., 1999]). After fertilization, putative zygotes were denuded at maximum vortex speed for 120 sec and transferred to a

modified SOF (SOF_{early}) supplemented with 10 μM EDTA, 0.5 mM fructose, 0.4 % (w/v) BSA FFA Fraction V, 0.1 mM taurine, 10 ng/ml epidermal growth factor (EGF; BD Biosciences—Discovery Labware, MA), without essential amino acids, and covered with light mineral oil for ~48 hr. Thereafter, cleavage rates were assessed and embryos were transferred to new droplets containing SOF_{mid1}, with essential and non essential amino acids, 0.4 % (w/v) BSA FFA Fraction V, 1.0 mM glucose, 10 ng/ml EGF, 100 μM β mercapto ethanol (β-ME), 1 μM EDTA and 1 ng/ml progesterone (P4) for ~48 hours. Embryos were transferred to fresh SOF_{mid2} droplets, which consists of SOF_{mid1} but supplemented with 1.5 mM glucose, under the same conditions for another ~48 hours. Finally, d-7 embryos were transferred for the last ~ 24 hours of culture to SOF_{late}, which is SOF supplemented with 5% (v/v) FCS, 0.1 mM taurine, 10 ng/ml EGF, 1 ng/ml P4 and 2.0 mM glucose.

Staining procedures

All incubations and washing steps were accomplished in 96-well dishes. Embryos were washed three times in 1x PBS supplemented with 0.1% PVP before transfer to the fixative solution. Embryos were fixed in 4% paraformaldehyde overnight at 4 °C. Subsequently, embryos were washed 3 times in PBS/PVP solution and incubated with the corresponding dye as follows. For neutral lipid staining, Nile red (Invitrogen, Molecular probes) was used at a concentration of 5 μg/ml of PBS/PVP and incubated at room temperature for 30 min in the dark. For cytoskeleton staining, the procedures and classification were based on Makarevich et al. (2011). Briefly, embryos were incubated overnight in a PBS/PVP solution supplemented with

0.1% Triton x-100 and stained with rodhamine phalloidin that selectively binds to Actin filaments (Invitrogen, Molecular probes) at a 1:20 dilution in PBS/PVP at room temperature for 30 min in the dark. Grade 1= F-Actin reticulated-well defined bands, grade 2= F-Actin bands fairly visible and grade 3= F-Actin bands poorly defined. For blastomere count, embryos were incubated in a PBS/PVP solution containing 10 μg/ml of Hoechst 33258 for 15 min at room temperature in the dark. Stained embryos were washed 3 times in PBS/PVP to reduce non-specific binding. Embryos were mounted on slides with 2 etched 10 mm diameter circles surrounded by white ceramic ink and covered with ProLong Gold antifade reagent (Invitrogen, Molecular probes).

Epifluorescent microscopy

All slides were visualized using a microscope (Imager Z1; Carl Zeiss, Inc.) equipped with 20X 0.5 NA ECPlan Neofluar objective (Carl Zeiss, Inc.). Embryo samples were excited at 340 nm, 488 nm and 540 nm and the emitted wave lengths were 470 nm, 515 nm and 565 nm to visualize DAPI nuclear stain, neutral lipids (Nile red) and cytoskeleton (F-actin), respectively. Images were captured with a cooled charged-coupled device camera (AxioCam MRm; Carl Zeiss, Inc.) and processed using AxioVision software (version 4.7.2; Carl Zeiss, Inc.).

BSA:CLA—Complex

The CLA isomer, *cis*9, *trans*11 (> 90% pure), was purchased from Nu-Chek Prep., Inc., Elysian MN. The CLA was conjugated to fatty acid-free BSA in a 4:1 ratio to prepare 4.5 mM CLA—BSA stocks using a method adapted from Keating et al. (2006).

Statistical analysis

One-way analysis of variance (ANOVA) between treatments was performed using JMP version 10 (Statistical Discovery from SAS). If replicates were different over time, we included replicate in our models as a random variable to control for the differences between and within experiments. If the main effect was significant, all means were compared using Tukey HSD. A probability of $P < 0.05$ was considered statistically significant and $P \leq 0.1$ was considered a trend. Proportional data not being normally distributed was arc-sine transformed and results are presented in the tables as back transformed data. Likewise, numerical data not being normally distributed was transformed to its natural logarithm. For the triglyceride content analyses, average pixel intensity from the captured Nile red stain images were obtained using the polygon selection tool to measure the whole embryo area (ImageJ 1.44p; Wayne Rasband National Institute of Health, USA). In order to assure that differences in intensity were from the triglyceride content *per se* and not due to the size of the embryo, we analyzed the differences in only one stage of embryo among experiments i.e. expanded stage.

RESULTS

The effects of PES and DNP supplementation on bovine embryos.

The results for this experiment are presented in Table 1; embryo cleavage rates were not different among treatments and remained above 80%. For development to blastocyst, there was a significant benefit from the combination of both PES and DNP as compared to control or treatment with either chemical alone (> 40% vs. ~30%;

main effect P = 0.01). Likewise, when the expanded-stage blastocyst rate was analyzed, the difference was 2-fold higher (P < 0.01) with the combined treatment than control Analyses of neutral lipid status (MPI values, Figure 1) showed a marked reduction in triglyceride content with metabolic regulators (main effect P = 0.04). PES or DNP alone reduced the triglyceride content by 20%. Total blastomere cell counts following treatment with metabolic regulators are illustrated in Figure 2. PES treatment resulted in a significantly reduced average number of blastomeres when compared to DNP or the combination. Embryonic morphological appearance indicated that PES alone resulted in delayed development and yielded blastocysts of reduced quality (Figure 3).

The effects of the combination of low PES and varied concentrations of DNP on bovine embryo development.

Similar to the previous experiment, embryo cleavage rates were not different among groups and remained above 80% (Table 2). Combination of DNP at 5 and 30 μM with PES resulted in numerically higher blastocyst rates (P = 0.06) compared with the control group (34 % and 38 % vs. 32 %, respectively). However, the positive control (DNP 10 μM, PES 0.3 μM) was consistently higher than the control group and values remained similar to the previous experiment (43 %). When blastocyst rates as a percentage of morula rates were analyzed, to account for the effect of adding the metabolic regulators at this stage, no significant differences were observed and the pattern of results remained similar (P = 0.06; Table 2).

Addition of metabolic regulators resulted in overall decreased MPI values as an indicator of neutral lipid content (main effect P = 0.04). The control group showed an MPI of 678 units that is very close to control values in Experiment 1. Embryos exposed to PES 0.3 μM and DNP 10 μM (Control +) showed a numerical reduction in lipid content by 5.47% when compared to the control group. The effect of treatment with combinations of PES 0.15 μM with DNP 5 to 30 μM was biphasic: a significant reduction in MPI of 8.6 % and 10.6 % relative to control for 5 and 10 μM , respectively (Figures 4), but the group with PES 0.15 μM and DNP 30 μM was higher and not different than the control group.

There was an overall negative effect on the blastomere number of expanded embryos when the higher concentrations of DNP were used (main effect P < 0.01). Combinations of PES 0.15 μM with DNP 10 and 30 μM resulted in reduced blastomere numbers compared with the cumulative average from control, control + and treatment with PES 0.15 μM and DNP 5 μM (99 vs. 121, respectively; Figure 5).

The effects of PES and DNP in combination with CLA *cis*9, *trans*11 on post-thaw viability of vitrified embryos.

Embryo cleavage rates were higher for the control group compared to the other treatments (80% vs. 75%; P = 0.03), but morula and total blastocyst rates were not significantly different. Comparing blastocyst rates as a percentage of morula rates, there was a trend for an effect of metabolic regulators combined as compared with other treatments (60% vs. 48%; P = 0.09; Table 3).

The effect of metabolic regulators in combination with CLA was tested on embryos undergoing vitrification procedures. Amongst all groups, embryos treated with metabolic regulators and without CLA resulted in a similar re-expansion rate as compared to control embryos after thawing (71% vs. 60%). CLA treated embryos showed the lowest re-expansion rates and this effect was more evident with the highest CLA dose (CLA 50 μM = 51%, CLA 100 μM =32%; Figure 6). Analyses regarding cytoskeleton integrity of the vitrified embryos showed that the distribution of the cytoskeleton integrity remained consistent among groups and cytoskeleton integrity of most embryos regardless of treatment were grade I and II (Figures 7 and 8). Blastomere counts were significantly different across treatment groups compared to control (130 ± 5 vs. 114 ± 6; P = 0.03).

DISCUSSION

Soon after fertilization, pre-implantation embryos undergo a series of metabolic adaptations in vivo in transit to the uterus. These adaptations include production of ATP from different substrates according to each of the corresponding microenvironments they encounter prior to implantation. Despite the reported inferior quality when compared with in vivo derived embryos, *in vitro* produced embryos show a remarkable plasticity for being able to develop in a wide variety of culture conditions. In vitro, a population of bovine embryos usually becomes arrested at the 16-32 cell stage and it has been suggested that this arrest originates by an alteration of glucose metabolism (Thompson, 2000). A low to high glucose metabolic switch occurs concomitantly as the embryo develops and reaches the morula stage with

corresponding metabolic adjustments suitable for a relatively hypoxic environment in the uterus. We attempted to circumvent the presumptive metabolic alteration with embryos becoming arrested by testing two glucose consumption enhancers that act on two different glucose pathways viz. glycolysis and pentose phosphate pathways (Thompson et al., 2000; De La Torre-Sanchez et al., 2006). An additive effect was observed when the two metabolic regulators where provided simultaneously during culture. We observed not only a ~10% increase in the total blastocyst rates, but also about twice as many expanded-stage embryos developed by day 8 after fertilization. Another benefit from metabolic regulator supplementation was the reduction in embryo triglyceride content compared to the control group. Embryos produced in vitro are often negatively affected by higher lipid content and, thus, these chemicals may provide another advantage to in vitro embryo production. Therefore we continued using that particular dose as our positive control for subsequent experiments. Once we observed that PES alone yielded lower results, we decided to reduce the dose for the subsequent experiments. One particular observation was that only a specific combination of MR resulted in beneficial effects. i.e. DNP 10 μM and PES 0.3 μM (1:33). When different proportions were used, results were not optimal. These results could be interpreted as further enhancing glycolysis by providing more DNP did not have a beneficial effect when the pentose phosphate pathway was not also further enhanced by PES in a way that glucose could be partitioned adequately. Interestingly reduction of triglyceride content in treated embryos with 0.15 μM PES and 5-30 μM DNP was biphasic and the highest combined dose of metabolic regulators resulted in similar triglyceride content as control. Although, in general, the reduction in

triglyceride levels with the metabolic regulators was encouraging, some of those embryos showed lower blastomere counts than embryos being supplemented with lower doses and control. Nonetheless, we also observed that among all treatments, 10 μM DNP and 0.3 μM PES yielded more embryos with better morphological appearance and this effect was consistent throughout all the experiments. Thus, this treatment combination not only improved blastocyst rates, but also improved embryo quality. Further, when we used several dose combinations of the metabolic regulators we mostly observed reductions in blastomere counts.

Based on our previous work (Absalón-Medina et al., 2011; Chapter Three) with embryo development and vitrification, we decided to follow-up with CLA supplementation and used two different doses of CLA, 50 μM and 100 μM, in combination with our best MR treatment. Our intention was to move forward and supplement CLA at a time relevant for embryo transfers and a more practical approach to benefit practitioners and producers using frozen embryos. Surprisingly, after vitrification procedures, embryos that were supplemented with CLA showed a substantial reduction in re-expansion rates after thawing, although, cytoskeletal integrity remained intact. Our data analysis on cytoskeleton integrity, accounting for F-Actin filaments staining, showed no substantial differences across all the treatments. We do not have a definitive explanation for this phenomenon; however, we suggest that CLA affected a mechanism for cyroprotectant-water exchange after thawing. Thus, embryos were not able to rehydrate the blastocoel cavity as normally occurs during the re-expansion process in expanded-stage blastocysts. We suggest that possible disruption of a mechanism of water transport would be most logical. We also

speculate that further culture would have provided additional time for those embryos to re-expand, but in our study all embryos were fixed immediately after thawing. Indeed, Kader et al. (2009) and Shu et al. (2009) reported that vitrified human blastocysts are cultured for additional hours in order to provide greater chance for rehydration to occur. Our previous report (Absalón-Medina et al., 2011) on CLA benefits were observed for embryos treated at the morula to early blastocyst transition stage. Therefore, the current expanded-stage embryos were at a different embryonic stage than the earlier embryos prior to development of a blastocoel cavity.

CONCLUSION

Addition of two metabolic regulators, PES and DNP in combination, showed a significant improvement in overall blastocyst rates, but most importantly improved expanded stage embryo rates. CLA treatment did not result in a further additive effect on embryo rates or in benefits after vitrification procedures and re-expansion rates were reduced. However, depending on dose, MR can reduce triglyceride content and yield a comparable good performance to withstand the vitrification procedures compared with control. Future studies are needed on the use of these metabolic regulators in combination with other important media components to emulate the natural occurring glucose kinetics in pre-implantation embryos in vitro.

ACKNOWLEDGMENTS

The authors would like to thank the Consejo Nacional de Ciencia y Tecnología (CONACyT, México) as well as the Department of Animal Sicence (Cornell University) for providing financial support to VAA during his PhD program. Thanks

to Cargill Wyalusing, PA for providing ovaries. We also appreciate the kind support from Genex Cooperative Inc., Ithaca, NY for donating semen. Thanks to Dr. Cohen's lab for providing access to and advice for epifluorescence microscopy. Special thanks to Professors R. Gilchrist and J. Thompson University of Adelaide, Australia for providing assistance in the establishment of SPOM in our laboratory.

REFERENCES

Absalón Medina, V. A., S. J. Bedford Guaus, R. O. Gilbert, L. C. Siqueira, G. Esposito, A. Schneider et al. 2011. Effect of conjugated linoleic acid supplementation on *in vitro* bovine embryo production and cryopreservation. american dairy science association (ADSA) joint meeting at new orleans, USA. J. anim. sci. vol. 89, E-suppl. 1/J. dairy sci. vol. 94, E-suppl. 1. (Abstr.).

Albuz, F. K., M. Sasseville, M. Lane, D. T. Armstrong, J. G. Thompson and R. B. Gilchrist. 2010. Simulated physiological oocyte maturation (SPOM): A novel in vitro maturation system that substantially improves embryo yield and pregnancy outcomes. Hum. Reprod. 25:2999-3011.

De La Torre-Sanchez, J. F., D. K. Gardner, K. Preis, J. Gibbons and G. E. Seidel Jr. 2006. Metabolic regulation of in vitro-produced bovine embryos. II. effects of phenazine ethosulfate, sodium azide and 2,4-dinitrophenol during post-compaction development on glucose metabolism and lipid accumulation. Reprod. Fertil. Dev. 18:597-607.

Gardner, D. K. and P. L. Wale. 2013. Analysis of metabolism to select viable human embryos for transfer. Fertil. Steril. 99:1062-1072.

Holm, P., P. J. Booth, M. H. Schmidt, T. Greve and H. Callesen. 1999. High bovine blastocyst development in a static in vitro production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology. 52:683-700.

Kader, A., A. Choi, Y. Orief and A. Agarwal. 2009. Factors affecting the outcome of human blastocyst vitrification. Reproductive Biology and Endocrinology. 7:99.

Keating, A. F., J. J. Kennelly and F. Q. Zhao. 2006. Characterization and regulation of the bovine stearoyl-CoA desaturase gene promoter. Biochem. Biophys. Res. Commun. 344:233-240.

Makarevich, A. V., E. Kubovicova, Z. Hegedusova, J. Pivko and F. Louda. 2011. Post-thaw culture in presence of insulin-like growth factor I improves the quality of cattle cryopreserved embryos. Zygote. 1-6.

Marei, W. F., D. C. Wathes and A. A. Fouladi-Nashta. 2009. The effect of linolenic acid on bovine oocyte maturation and development. Biol. Reprod. 81:1064-1072.

Ménézo, Y., I. Lichtblau and K. Elder. New insights into human pre-implantation metabolism in vivo and in vitro. 2013. JARG. 30: 293-303.

Parrish, J. J., J. Susko-Parrish, M. A. Winer and N. L. First. 1988. Capacitation of bovine sperm by heparin. Biol. Reprod. 38:1171-1180.

Parrish, J. J., J. L. Susko-Parrish and N. L. First. 1989. Capacitation of bovine sperm by heparin: Inhibitory effect of glucose and role of intracellular pH. Biol. Reprod. 41:683-699.

Pereira, R. M., I. Carvalhais, J. Pimenta, M. C. Baptista, M. I. Vasques, A. E. Horta, I. C. Santos, M. R. Marques, A. Reis, M. S. Pereira and C. C. Marques. 2008. Biopsied and vitrified bovine embryos viability is improved by trans10, cis12 conjugated linoleic acid supplementation during in vitro embryo culture. Anim. Reprod. Sci. 106:322-332.

Shu, Y., J. Watt, J. Gebhardt, J. Dasig, J. Appling and B. Behr. 2009. The value of fast blastocoele re-expansion in the selection of a viable thawed blastocyst for transfer. Fertil. Steril. 91:401-406.

Sudano, M. J., D. M. Paschoal, S. Rascado Tda, L. C. Magalhaes, L. F. Crocomo, J. F. de Lima-Neto and C. Landim-Alvarenga Fda. 2011. Lipid content and apoptosis of in vitro-produced bovine embryos as determinants of susceptibility to vitrification. Theriogenology. 75:1211-1220.

Thompson, J. G. 2000. In vitro culture and embryo metabolism of cattle and sheep embryos - a decade of achievement. Anim. Reprod. Sci. 60-61:263-275.

Thompson, J. G., C. McNaughton, B. Gasparrini, L. T. McGowan and H. R. Tervit. 2000. Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured in vitro. J. Reprod. Fertil. 118:47-55.

TABLES

Table 1. The effects of PES and DNP supplementation on bovine embryos.

Group	Total oocytes	Cleavage %	Blastocyst[1]%	Ex blastocyst %
Control	39.8 ± 0.9	79.7 ± 2.7	31.5 ± 2.7[b]	15.1 ± 2.3[b]
PES (0.3 μM)	38.6 ± 0.9	80.3 ± 2.7	33.8 ± 2.6[b]	16.7 ± 2.2[b]
DNP (10 μM)	38.4 ± 1.0	80.5 ± 2.8	32.2 ± 2.8[b]	17.5 ± 2.4[b]
PES + DNP	39.7 ± 1.1	86.1 ± 3.3	42.8 ± 3.2[a]	27.9 ± 2.7[a]

[*]mean ± SEM from 8 replicates. Means within a column not sharing the same letter are significantly different ($P < 0.05$).
[1]Blastocyst and expanded (Ex) blastocyst rates were based on percentages of the original oocyte number.

Table 2. The effects of the combination of low PES and varied concentrations of DNP on bovine embryos.

	Original number of COCs					Original number of morulas	
Group	Total oocytes	Cleavage %	Morula %	Blastocyst[1] %	Ex blastocyst[1] %	Blastocyst[2] %	Ex blastocyst[2] %
Control	42.3 ± 0.7	80.7 ± 2.9	72.4 ± 3.0	32.0 ± 3.3	15.5 ± 2.9	44.4 ± 3.7	21.41 ± 3.5
Control +	41.8 ± 0.6	85.5 ± 3.1	77.6 ± 3.3	42.5 ± 3.6	21.8 ± 3.2	54.3 ± 4.1	27.68 ± 3.9
[3]One	41.0 ± 0.6	82.3 ± 2.9	73.4 ± 3.0	30.5 ± 3.3	17.5 ± 2.9	41.2 ± 3.7	23.90 ± 3.6
[4]Two	41.5 ± 0.7	80.9 ± 2.9	73.3 ± 3.0	34.3 ± 3.3	17.3 ± 2.9	47.5 ± 3.6	24.08 ± 3.5
[5]Three	41.5 ± 0.7	84.6 ± 2.9	74.9 ± 3.0	38.2 ± 3.3	21.6 ± 2.9	50.4 ± 3.7	28.32 ± 3.5

[*]mean ± SEM from 6 replicates. Means within columns were not significantly different.
[1]Blastocyst and expanded (Ex) blastocyst rates were based on percentages of the original oocyte number.
[2]Blastocyst and expanded (Ex) blastocyst rates were based on percentages of the original morula number.
[3]Embryos treated with 0.15 μM PES and 5 μM DNP.
[4]Embryos treated with 0.15 μM PES and 10 μM DNP.
[5]Embryos treated with 0.15 μM PES and 30 μM DNP.

Table 3. The effects of metabolic regulators in combination with CLA *cis*9, *trans*11 on embryo development.

Group	Total oocytes	Cleavage %	Morula %	Blastocyst[1] %	Blast[2] % Mor
Control	42.3 ± 1.4	80.7 ± 0.9[a]	70.6 ± 2.3	33.1 ± 1.9	47.0 ± 2.8
[3]MRCLA 50	42.0 ± 1.5	76.9 ± 1.2[b]	63.1 ± 3.2	31.8 ± 2.6	50.8 ± 3.9
[4]MRCLA 100	41.7 ± 1.5	76.8 ± 1.3[b]	66.4 ± 3.2	31.9 ± 2.6	48.2 ± 3.9
[5]Met Reg	40.6 ± 1.6	75.9 ± 1.5[b]	63.8 ± 3.9	38.4 ± 3.2	60.2 ± 4.7

[*]mean ± SEM from 3 replicates. Means within a column not sharing the same letter are significantly different ($P < 0.05$).
[1]Blastocyst rates were based on percentages of the original oocyte number.
[2]Blastocyst rates were based on percentages of the original morula number.
[3]Embryos treated with 0.3 μM PES and 10 μM DNP (MR) plus 50 μM CLA.
[4]Embryos treated with 0.3 μM PES and 10 μM DNP (MR) plus 100 μM CLA.
[5]Embryos treated with 0.3 μM PES and 10 μM DNP.

FIGURES

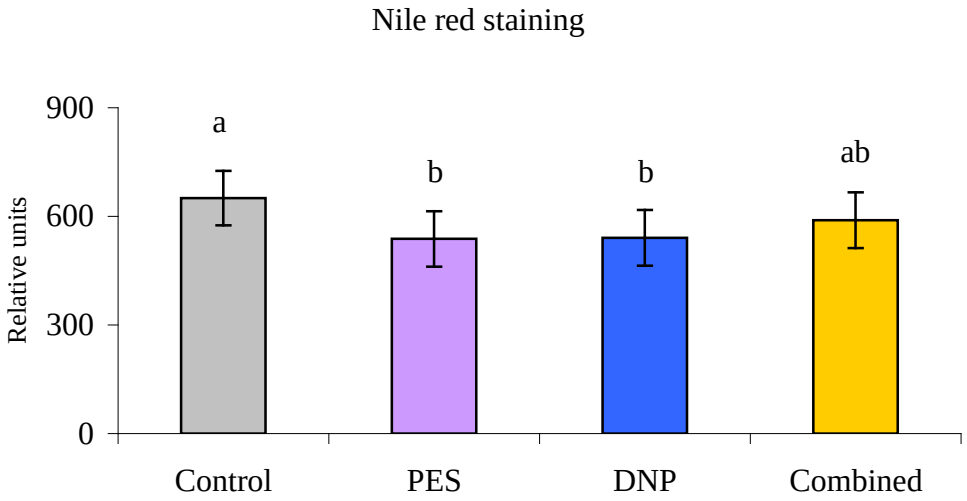

Figure1. The effect of metabolic regulators on triglyceride content (mean pixel intensity, MPI).

PES= 0.3 μM DNP= 10 μM. Means ± SEM not sharing the same letter are significantly different. A total of 64 expanded-stage blastocyts were used.

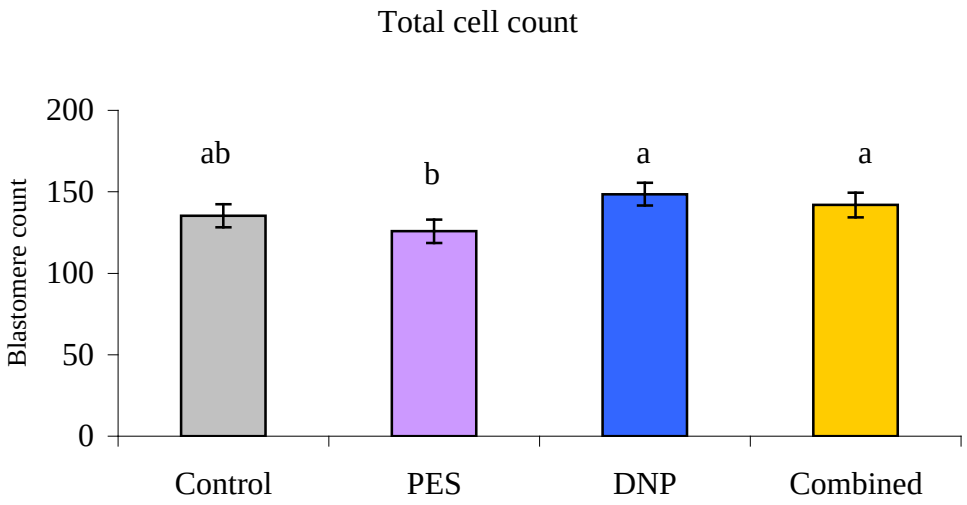

Figure 2. The effect of metabolic regulators on embryo blastomere cell count.

PES= 0.3 μM DNP= 10 μM. Means ± SEM not sharing the same letter are significantly different. A total of 66 expanded-stage blastocyts were used.

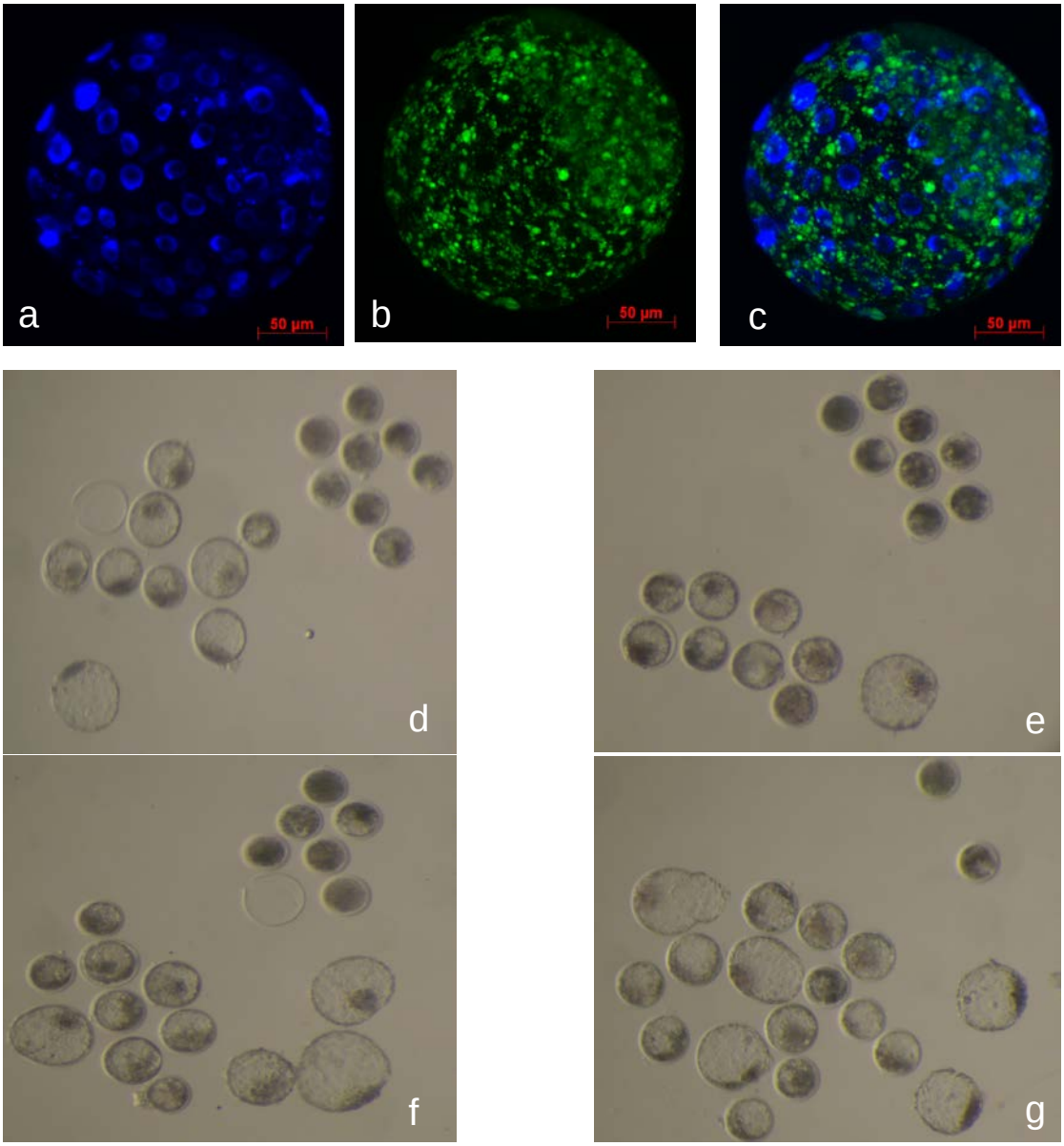

Figure 3. The effect of metabolic regulators on embryo development and quality.

Panels a, b and c show blastomere nuclei, Nile red-stained lipids and merged images, respectively. Panel d = control embryos; Panel e = PES treated embryos; Panel f = DNP treated embryos; Panel g = PES+DNP treated embryos in experiment 1.

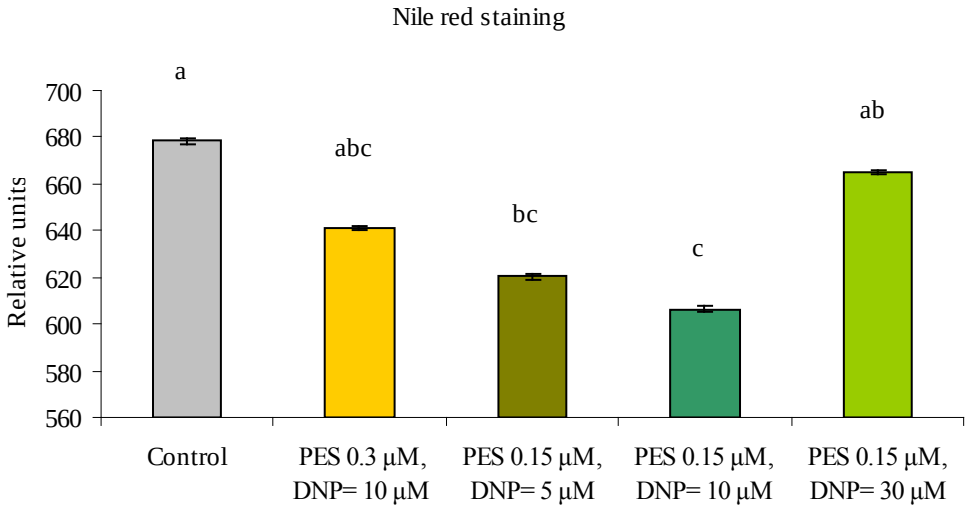

Figure 4. The effect of different concentrations of metabolic regulators on embryo triglyceride content (Pixel Intensity (MPI) measurements).

Control + =PES 0.3 µM, DNP= 10 µM.
One = PES 0.15 µM, DNP 5 µM
Two = PES 0.15 µM, DNP 10 µM
Three = PES 0.15 µM, DNP 30 µM
Means ± SEM not sharing the same letter are significantly different. A total of 220 expanded-stage blastocyts were used.

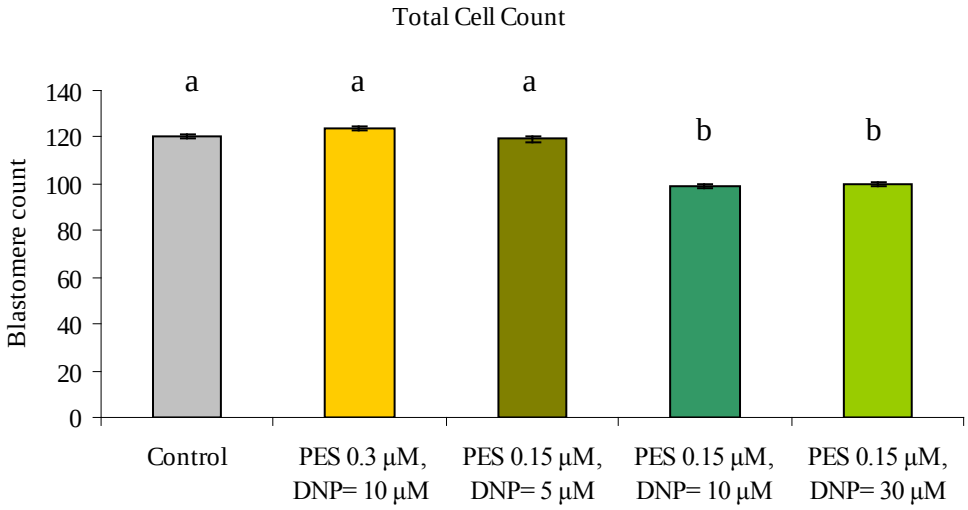

Figure 5. The effect of different concentrations of metabolic regulators on embryo blastomere cell count.

Control + =PES 0.3 µM, DNP= 10 µM.
One = PES 0.15 µM, DNP 5 µM
Two = PES 0.15 µM, DNP 10 µM
Three = PES 0.15 µM, DNP 30 µM
Means ± SEM not sharing the same letter are significantly different. A total of 208 expanded-stage blastocyts were used.

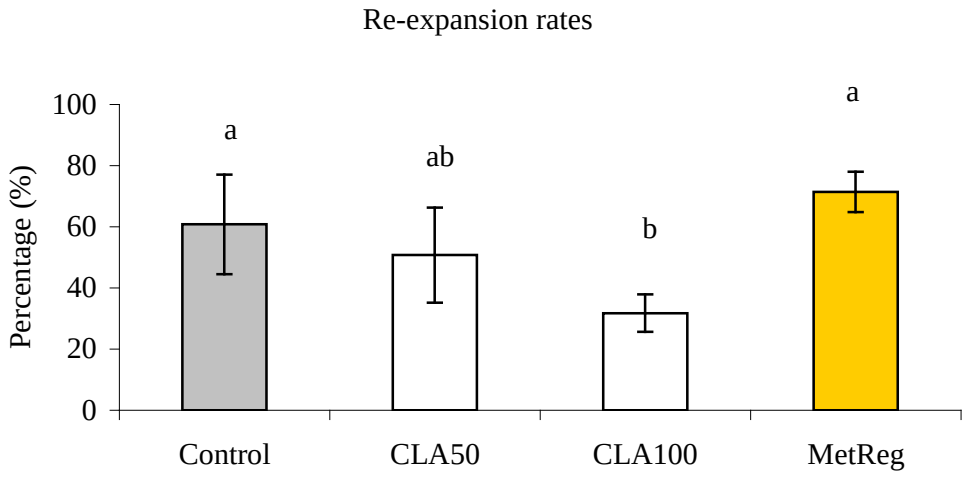

Figure 6. The effect of metabolic regulators and CLA on re-expansion rates post-thaw of vitrified expanded stage embryos at day 8 post IVF.

Control group was vitrified and there was no other treatment. CLA50 or CLA100 groups= embryos treated with 50 μM or 100 μM CLA + MR. MetReg group= PES 0.3 μM + DNP 10 μM. Means not sharing the same letter are significantly different. A total of 93 embryos were used.

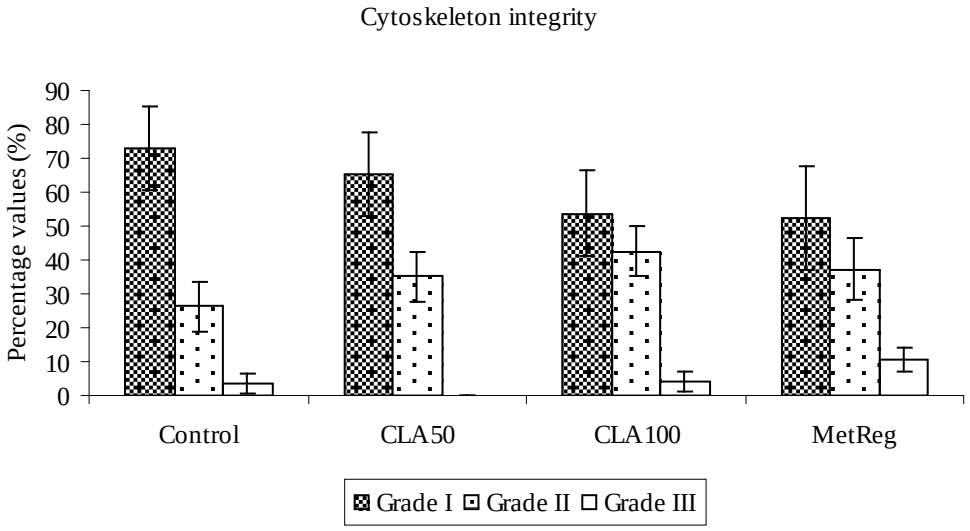

Figure 7. The effect of metabolic regulators and CLA on cytoskeleton integrity.

Among groups, most embryos showed defined F-Actin bands and fell into grade I and II categories. There were no statistical differences within categories. A total of 93 embryos were used.

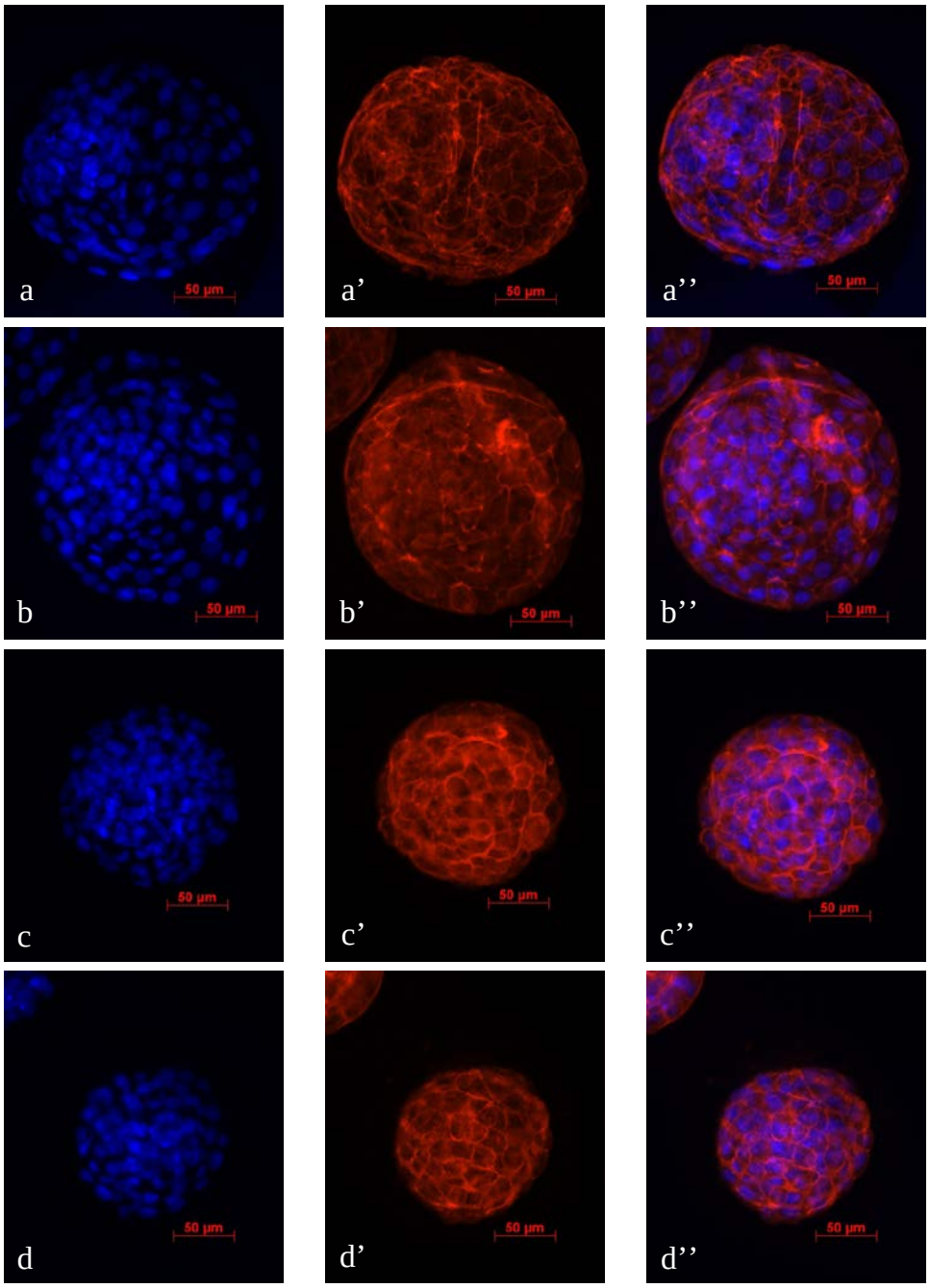

Figure 8. The effect of metabolic regulators and CLA on embryo quality after vitrification procedures.

This figure shows only grade I (vide Figure 7) F-Actin bands. Panel a shows Control group. Panel b shows MetReg group. Panel c shows MR + CLA50 µM group and panel d shows MR + CLA100 µM group. Letter alone shows nuclear Hoechst stain. Letter + an apostrophe show F-Actin stain and letter + two apostrophes shows the merged image.

CHAPTER FIVE: COMPARISON OF SEVERAL FRACTIONATION METHODS ON SPERM MOTILITY AND *IN VITRO* BOVINE EMBRYO DEVELOPMENT WITH CONVENTIONAL OR SEX-SORTED SEMEN.

V. A. Absalón-Medina[†], P. Hung[‡], S. H. Cheong[‡], S. S. Suarez[‡], R. O. Gilbert[‡], and W. R. Butler[†*]

In final preparation for submission to Reproduction in Domestic Animals

[†]College of Agriculture and Life Sciences, Animal Science Department. Cornell University, Ithaca, NY 14853. USA.

[‡]College of Veterinary Medicine, Department of Clinical Sciences. Cornell University, Ithaca, NY 14853. USA

[*]Correspondence e-mail: wrb2@cornell.edu

ABSTRACT

The use of in vitro fertilization has been gradually increasing during the past decade. It seems likely that, in bovine species, this assisted reproductive technology (ART) will be the method of choice in the near future since it has several advantages over conventional breeding methods, especially for gender-selected semen. The use of colloidal macromolecules in sperm fractionation methods is required for IVF to eliminate bad quality sperm and inherent bacterial contamination. The objectives of this study were to compare four different colloidal-based sperm separators i.e. Percoll density gradient centrifugation (DGC), Old Bovipure single layer centrifugation (SLC), New Bovipure (SLC) and Androcoll-B (SLC) on computerized sperm motility parameters and to evaluate the effects on in vitro embryo development. The second objective was to evaluate the effects of metabolic regulators (MR) in vitro on embryo development of gender-selected embryos.

Semen purified with New Bovipure and Androcoll-B showed numerically higher velocity and linear motility values than the other two treatments, but Androcoll-B treated sperm displayed a lower hyperactivation profile compared with other treatments. Overall, sperm motility parameters were positively correlated with blastocyst development across treatments except for group Old Bovipure.

Addition of metabolic regulators to embryos originated from X-sorted sperm for IVF had reduced development from morula to blastocyst stages, and those reaching expanded blastocyst stages presented a smaller blastocoele when compared with untreated embryos. Blastomere counts were reduced in the MR treated embryos.

In conclusion, New Bovipure and Androcoll-B resulted in improved computerized motility parameters that were positively correlated with embryo development. Treatment with metabolic regulators PES and DNP in combination reduced overall blastocyst rates of X-sorted IVF embryos. Future studies are needed on the use of these metabolic regulators in combination with other additives to determine the gender-specific metabolic requirements of embryos.

INTRODUCTION

In several countries around the globe, dairy cattle reproduction relies mostly on ovulation synchronization and timed artificial insemination with frozen semen. In the bovine semen industry, entire ejaculates are typically processed with egg yolk or milk-based extenders before freezing and storage. Usually, after semen is extended it contains a heterogeneous pool of spermatozoa including immature, abnormal and damaged sperm. In addition, any bacteria, cellular debris and leukocytes present may be a potential source of reactive oxygen species (ROS) and these are all detrimental factors, especially in an in vitro fertilization (IVF) context (Morrell, 2006). However, because acceptable post-thaw sperm performance assessed by computerized motility parameters can usually be attained, alternative sperm preparation methods such as sperm-density gradient centrifugation is not used in the semen industry. The current system for processing frozen semen stems from the fact that sire selection criteria include sperm survival and performance as contributions towards the sire's overall fertility. Thus, sires with non-viable sperm after freezing are more likely to be culled. Nonetheless, selection for sires that produce sperm with good *freezability* traits may have resulted inadvertently in selection of undesirable genetic traits that reduce fertility and is a current management issue in dairy cattle (Morrell and Rodriguez-Martinez, 2011; Soggiu et al., 2013).

In human and endangered species with sub-optimal sperm populations, semen is highly valuable and discarding poor specimens is not an option. It has been reported in human IVF that processing the semen by density gradient centrifugation has resulted in increased conception rates (Morrell, 2006). Sperm density gradient separators, based on macromolecules suspended in a colloidal solution, are designed to sort motile

spermatozoa with normal morphology and intact DNA from the total sperm population, hence discriminating against immature and poor quality spermatozoa. During colloidal-based sperm separation procedures, either by density gradient (DGC) or single layer centrifugation (SLC), viable sperm move to a point in the gradient that matches their own density i.e. iso-pynic point (Pertoft, 2000). Thus, by separating out good quality sperm from a sample, and eliminating ROS and bacteria, semen is better able to withstand the stress effects of cryopreservation procedures and to have a longer survival time, thereby, to maximize potential for optimal fertilization (Morrell, 2000; Morrel and Rodriguez-Martinez, 2011).

Among the many sperm separation methods that have been developed to improve sperm quality, DGC and swim-up are the most commonly used (Samardzija et al., 2006). Historically, Percoll DGC has been used as the method of choice to efficiently separate out sperm for human IVF procedures (Pertoft et al. 1978). Nevertheless, it has been reported in some studies (De Vos et al., 1997; Carrell et al., 1998) that Percoll has a toxic effect on human sperm, while not in others (Scott and Smith, 1997; Matás et al., 2003). Other studies indicated that there was a batch to batch variability in the degree of endotoxin levels present in the product and this led to the withdrawal of Percoll for human IVF by Pharmacia Biotech in 1996 (Classens et al., 1998; Moussett-Siméon et al., 2004). Semen quality and related issues led researchers to explore alternative products for DGC that display minimal variability from batch to batch and reduce the risk of increasing endotoxin levels.

Alternative sperm fractionation products that are currently used for bovine species are Bovipure (Nidacon International AB, Göthenborg, Sweden) and Androcoll-B

(Swedish University of Agricultural Sciences, Uppsala, Sweden). Both products are silane-coated silica particles designed specifically to account for the density of viable sperm. In addition, according Nidacon's recommendations, Bovipure can be used for both DGC and SLC. Further, Nidacon has recently improved their original formula Bovipure Bottom and Bovipure Top (Old Bovipure). The current version of Bovipure 100 (New Bovipure) comes with a diluent. Androcoll-B is a ready-to-use solution with a proprietary formula owned by Dr. Morrell from the Swedish University of Agricultural Sciences, Uppsala, Sweden. A study by Samardzija et al. (2006) compared Bovipure DGC to the swim-up method and sperm recovered by DGC outperformed swim-up treated sperm based on motility and morphology. Although there were no differences at embryo cleavage stage, day-7 blastocyst rates were higher in embryos originating from the Bovipure DGC treatment. When used for SLC, Androcoll-B has proven not only to be equally efficient in terms of sperm recovery, motility and embryo development in vitro, but also to be more practical than DGC when working with large-volume ejaculates (Thys et al., 2009).

Currently in the cattle industry it is highly desirable to generate offspring of a specific gender. In the dairy industry, heifers are preferred over bull calves because of the needs for herd replacement and milk production. Increased use of sex-sorted semen has stimulated a new line of investigation among research groups. Further, the potential of obtaining female embryos in vitro exists and producing animals with known gender provides producers with more options for heifer replacement programs. However, in order for the technology of IVF coupled with sex-sorted semen to be efficiently exploited, it is mandatory to identify critical substrates and additives to improve embryo

production in vitro. There are several obstacles to overcome before this technique provides consistent results and becomes fully adopted in an in vitro embryo production system. The first obstacle is sire selection based on sperm *sortability* traits similar to sperm freezability traits (*vide supra*) since there was high variation among sires in an in vitro fertilization study (Cran et al. 1993). Another obstacle is that there seems to be a sperm-specific heparin concentration requirement for capacitation/hyperactivation during IVF that varies among sires (Lu and Seidel, 2004; Xu et al., 2009). A third important obstacle for sex-sorted semen is to find the minimal sperm concentration per number of oocytes, given that the recovery of sperm after centrifugation becomes substantially lower than when conventional semen is used. Although it has been reported by Xu et al. (2006) that as few as 600 sperm per oocyte are needed for optimal in vitro fertilization, due to the large variation among bulls, a reliable standard minimum sperm concentration needs to be established. Lastly gender-selected embryos may present different metabolic requirements. This is an important area for investigation as sex-sorted semen IVF may become the method of choice based on the fact that many oocytes can potentially be fertilized with one semen-straw rather than inseminating one cow.

The first objective of the current study was to compare four different colloidal-based sperm separators: Percoll (DGC), Old Bovipure (SLC), New Bovipure (SLC) and Androcoll-B (SLC) on sperm motility parameters and to evaluate the effects on embryo development. The second objective was to evaluate the effects of metabolic regulators (MR) on in vitro development of gender-selected embryos.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents were obtained from Sigma-Aldrich Co. (Saint Louis, MO, USA), unless otherwise indicated.

Experimental design

The effect of four colloidal-based sperm separators on sperm motion parameters and embryo development in vitro with conventional semen.

In 3 replications, sperm analyses were performed soon after the last IVF well with oocytes was inseminated. One portion of each tube containing corresponding sperm from Percoll, Old Bovipure, New Bovipure and Androcoll-B treatments were analyzed by computer-assisted semen analysis (CASA). CASA uses 7 parameters to evaluate sperm motility.

In vitro embryo production was carried out in five replicates, and four groups of cumulus oocyte complexes (COCs) were inseminated with sperm recovered by the aforementioned separator treatments. Early cleavage (36 hr post IVF) and late cleavage (48 hr post IVF) rates were measured to evaluate sperm treatment effects on embryo development. In addition, morula and blastocyst rates were measured at d 5 and d 8 post IVF to evaluate any effect from sperm treatment on further preimplantation embryo development. Blastomere nuclei staining under epifluorescent microscopy was used to determine total blastomere cell counts. A total of 851 COCs were used in this experiment.

The effects of metabolic regulators on development of in vitro produced embryos using X-sorted semen.

In 4 replications, this trial measured the effects of the combined metabolic regulators, phenazine ethosulfate (PES) 0.3 μM and 3, 4—dinitrophenol (DNP) 10 μM, (Chapter Four) provided at morula stage on subsequent development of gender-selected female preimplantation embryos. Two groups were used: presumptive female-embryos originating from IVF with sex-sorted sperm were treated with or without MR at morula stage. In addition, embryos originating from IVF using conventional semen were used as internal control. Cleavage rates as well as morula and blastocyst rates were measured at days 2, 5 and 8 post IVF. Hoechst staining under epifluorescent microscopy was used to determine total blastomere cell counts. A total of 449 COCs were used in this experiment.

Oocyte recovery and selection

Bovine ovaries were collected at a nearby abattoir (120 km) and transported to the laboratory in prewarmed lactated Ringer's solution at 30-35 °C. Cumulus oocyte-complexes (COCs) from 2-8 mm follicles were aspirated with an 18G hypodermic needle attached to an aspiration pump unit adjusted to a flow of 22.5 ± 2.5 ml of H_2O per minute. Follicular fluid supernatant was removed and the pellet containing COCs was transferred to a 15 ml tube where it was resuspended in holding medium and the contents were poured gently into a 100 mm Petri dish. Holding medium consisted of TCM-199 Hank's salts (Invitrogen, Grand Island, NY), 10% Fetal Calf Serum (FCS; Invitrogen), 25 μg/ml of gentamicin, 0.2 mM Na-pyruvate, and heparin 5 μg/ml with a mOsm = 300 ± 1. Selection of COCs was based on morphological assessment as having several layers of

cumulus granulosa cells and oocytes with a homogenous cytoplasm. The entire process was performed within 5 hours, including transportation.

In vitro pre-maturation

Following the method of Albuz et al. (2010), selected COCs were pre-matured in holding medium supplemented with forskolin, an adenylate cyclase activator, and IBMX, a non-specific phosphodiesterase (PDE) inhibitor, at a final concentration of 100 μM and 500 μM, respectively, in order to increase overall cAMP levels and to inhibit overall PDEs in both cumulus cells and oocytes. Millimolar stock concentrations of the chemicals were stored at -20 °C dissolved in dimethylsulphoxide (DMSO) and used fresh in each trial. In groups of about 40 COCs, pre-maturation treatment was carried out under atmospheric and humidified conditions at 38.5 °C for 2 hours.

In vitro maturation

Selected COCs were matured in groups of 40 ± 5 for 30 hours in 400 μL of TCM-199 (Earle's Salts) enriched with 10% FCS, 0.2 mM sodium pyruvate, 1 mM alanyl-glutamine, 0.1 mM taurine, 0.1 mM cysteamine, 1 μg/ml estradiol, 85 mU/ml bovine follicle stimulating hormone (FSH, SIOUX Biochemical, Inc., Sioux Center, IA), 20 μg/ml gentamicin, at pH 7.35 ± 0.02 and mOsm 300 ± 2 and covered with light mineral oil in a humidified atmosphere at 38.5 °C with 5% CO_2 in air. In addition, based on Albuz et al. (2010), COCs were cultured during the entire maturation process with a type 3-specific PDE inhibitor, cilostamide (20 μM; Biomol, Plymouth Meeting, PA, USA).

In vitro fertilization

After a total of 30 hours, presumptive matured oocytes were transferred to a modified IVF medium (Fert-TALP; Parrish et al., 1988) supplemented with 0.5 mM fructose, 0.2 mM non-essential amino acids, 6 mg/ml BSA FFA Fraction V, 30 μM penicillamine, 15 μM hypotaurine, 1.5 μM epinephrine (PHE), 22 μg/ml heparin, 20 μg/ml gentamicin, covered with light mineral oil in a humidified atmosphere at 38.5 °C with 5% CO_2 in air for 18 hours (pH of 7.38 ± 0.01, mOsm 285 ± 1). Frozen semen straws (Genex, Ithaca NY, USA) from a single bull and from the same ejaculate were thawed at 37 °C for 30 seconds. Motile sperm were separated from cryoprotectant, non-motile sperm, and debris by the following methods: Percoll® DGC (90% and 45%), Old Bovipure (SLC), New Bovipure (SLC; Nidacon International AB, Göthenborg, Sweden) and Androcoll-B (SLC; Swedish University of Agricultural Sciences, Uppsala, Sweden) at 300 x g for 20 min. Subsequently, sperm were washed twice in 5 ml of Boviwash (Nidacon International AB, Göthenborg, Sweden) and centrifuged at 300 x g for 5 min to remove each corresponding colloid. Finally, sperm were adjusted to a final concentration of 1.5 x 10^6 sperm/ml using Fert-TALP media and applied to the oocytes. For sex-sorted semen experiments 5 straws were thawed and sperm was recovered by using the New Bovipure SLC method. After all centrifugations, 2-3 million sperm were recovered and the final concentration of sperm was adjusted to 2 x 10^6 sperm/ml Fert-TALP media.

In vitro embryo culture

A modified synthetic oviductal fluid (SOF) sequential media was used (20 μg/ml gentamicin, pH 7.4 ± 0.01, mOsm 275 ± 5, humidified atmosphere at 38.5 °C with 5% CO_2, 7% O_2, and 88% N_2 [Holm et al., 1999]). After fertilization, putative zygotes were

denuded at maximum vortex speed for 120 seconds and transferred to a modified SOF (SOF_{early}) supplemented with 10 μM EDTA, 0.5 mM fructose, 0.4 % (w/v) BSA FFA Fraction V, 0.1 mM taurine, 10 ng/ml epidermal growth factor (EGF; BD Biosciences—Discovery Labware, MA), without essential amino acids, and covered with light mineral oil for ~48 hours. Thereafter, cleavage rates were assessed and embryos were transferred to new droplets containing SOF_{mid1}, with essential and non essential amino acids, 0.4 % (w/v) BSA FFA Fraction V, 1.0 mM glucose, 10 ng/ml EGF, 100 μM β mercapto ethanol (β-ME), 1 μM EDTA, and 1 ng/ml progesterone (P4) for ~48 hours. Embryos were transferred to fresh SOF_{mid2} droplets, which consists of SOF_{mid1} but supplemented with 1.5 mM glucose, under the same conditions for another ~48 hours. Finally, d-7 embryos were transferred for the last ~ 24 hours of culture to SOF_{late}, which is SOF supplemented with 5% (v/v) FCS, 0.1 mM taurine, 10 ng/ml EGF, 1 ng/ml P4 and 2.0 mM glucose.

Staining procedures

Blastomere staining was based on Block et al. (2008). All incubations and washing steps were accomplished in 96-well dishes. Embryos were washed three times in PBS supplemented with 0.1% PVP (PBS/PVP) and then fixed in 4% paraformaldehyde PBS/PVP supplemented with 10 μg/ml Hoechst 33258 for 15 minutes at room temperature in the dark. Subsequently, embryos were washed 3 times in PBS/PVP solution to reduce non-specific binding. Embryos were mounted on slides with 2 etched 10 mm diameter circles surrounded by white ceramic ink and covered with ProLong Gold antifade reagent (Invitrogen, Molecular probes).

Epifluorescent microscopy

All slides were visualized using a microscope (Imager Z1; Carl Zeiss, Inc.) equipped with 20X 0.5 NA ECPlan Neofluar objective (Carl Zeiss, Inc.). Embryo samples were excited at 340 nm and the emitted wave length was 470 nm to visualize DAPI nuclear stain for the total cell count assessment. Images were captured with a cooled charged-coupled device camera (AxioCam MRm; Carl Zeiss, Inc.) and processed using AxioVision software (version 4.7.2; Carl Zeiss, Inc.).

Assessment of sperm motion parameters by computer-assisted semen analysis (CASA)

Immediately after IVF, 10 μL of sperm (3 replicates for each treatment) from the aliquots remaining were placed in a 2-chamber slide (Leja, The Netherlands) and sperm motion parameters were assessed by a Hamilton Thorne IVOS Sperm Analysis System (Hamilton Throne Inc., Beverly, MA). Smoothed path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), linearity (LIN), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), and straightness (STR) were determined. Instrument settings for the CASA analysis were as follows: frame rate 60 Hz; frames acquired 30, minimum contrast 70, minimum cell size 5 pixels, static VAP cutoff 5 μ/s, static VSL cutoff 15 μ/s, progressive VAP threshold 50 μ/s. Incomplete and crossed tracks were manually removed from data analysis. For each sample, at least 150 sperm were assessed.

Statistical analysis

One-way analysis of variance (ANOVA) among treatments was performed using JMP version 10 (Statistical Discovery from SAS). If replicates were different over time,

we included replicate in our models as a random variable to control for the differences between and within experiments. If the main effect was significant, all means were compared using Tukey HSD. Comparison of means between the two sex-sorted semen groups was accomplished by Student's t test. A probability of $P < 0.05$ was considered statistically significant and $P \leq 0.1$ was considered a trend. Proportional data not being normally distributed was arc-sine transformed and results are presented in the tables as back transformed data. Likewise, numerical data not being normally distributed was transformed to its natural logarithm.

RESULTS

The effect of several colloidal-based sperm fractionation methods on sperm motion parameters and embryo development in vitro with conventional semen.

We examined sperm motion parameters of the 4 colloidal-based sperm fractionation methods in this study to evaluate correlation of sperm motility performance with fertilization and embryo cleavage outcomes. Differences in sperm motion parameters among the 4 treatments (3 replicates each) in the study were observed and the results are summarized in Table 1. Sperm treated with New Bovipure and Androcoll-B tended to have higher velocity (VAP and VSL) and LIN motility values than those treated with Percoll or Old Bovipure. Sperm seemed to display one of the features of hyperactivation detected by high ALH (> 6.4 μM) even though the STR was above 90%. However, low ALH values were observed for Androcoll-B ($P < 0.05$) indicating that the sperm were not as hyperactive as the sperm from the other treatments.

The effects of Percoll (DGC), Old Bovipure (SLC), New Bovipure (SLC) and Androcoll-B (SLC) on embryo development were assessed. There was a treatment effect on early cleavage rates (main effect: $P = < 0.05$) with Percoll being higher than Androcoll-B (Figure 1). There was a trend for Androcoll-B group to show higher late cleavage rates when compared to the other groups ($P = 0.1$; 13 % vs. ≤ 6 %). Total cleavage rates among groups followed a similar pattern as early cleavage rates (Percoll yielded higher cleavage rate than Androcoll-B group.

Analyses of embryo development during continued culture showed no significant differences among groups assessed at morula stage (5 days post-IVF) and values were above 80% (Table 2). However, significant differences were observed among blastocyst

rates (main effect: $P < 0.05$) where New Bovipure treatment yielded the highest rate and Old Bovipure yielded the lowest blastocyst development rate in this experiment.

Blastomere counts were used to assess the developmental quality of expanded-stage embryos and no significant differences were detected among treatments (Percoll = 137 ± 5, Old Bovipure = 148 ± 6, New Bovipure = 149 ± 5 and Androcoll-B = 152 ± 7).

The effects of metabolic regulators on development of in vitro produced embryos using X-sorted semen.

The effect of MR provided in culture at day 5 post IVF on embryo development and quality of gender-selected female embryos was evaluated (Table 3). Cleavage rates and subsequent development to morula stage at day 5 post IVF were not significantly different between MR-treated and non-treated female embryos. Blastocyst rates assessed at day 8 post IVF showed a trend for untreated embryos to display higher values than those treated with MR. When blastocyst rates were evaluated at specific stages of development, untreated embryos displayed higher expanded stage (30 % vs. 17 %) as well as hatched stage (5% vs. 0%) embryos when compared to MR treated female embryos ($P < 0.05$; Figure 2). The MR-treated female embryos showed reduced blastomere counts compared to untreated embryos (Figure 3; $P < 0.05$). All experiments were carried out in parallel with a quality control group using conventional semen and results fell within the historical range based on previous experiments (data not shown). Figure 4 presents pictures of embryo morphology for all groups.

DISCUSSION

Over the past 20 years the use of IVF has been gradually increasing and, presumably in the bovine species, this ART will become the method of choice, because it has several advantages over conventional breeding methods such as multiple ovulation embryo transfer (MOET) programs. For example, using ovum pick-up (OPU) coupled with IVF, oocytes can be harvested from cows as early as 30 days after calving that is substantially shorter than the waiting period for a cow to enter into a MOET program occurring somewhat later post-calving. Moreover, twice as many embryos can be produced in an OPU/IVF system compared with MOET programs. In addition, as the use of sex-sorted semen has the potential to yield better results when coupled with IVF technology, the capability to produce gender-selected embryos would open new opportunities both for research and business interests. In order for this to become a reality, it is necessary to optimize methods for the recovery of elite-quality sperm to improve current IVF outcomes. In addition, as embryos would be produced by gender it will be necessary to define not only sex-specific, but also stage-specific embryo metabolic requirements during culture.

The first objective of the current study was to compare four different colloidal-based sperm fractionation methods i.e. Percoll (DGC), Old Bovipure (SLC), New Bovipure (SLC) and Androcoll-B (SLC) on sperm motility parameters and to evaluate those effects on embryo development. Alternative colloidal-based sperm fractionation methods resulted in improved computerized motility parameters that were positively correlated with embryo development. In experiment 1, it was observed that sperm separated with New Bovipure and Androcoll-B showed higher velocity and linear

motility values as compared to the other treatments. Interestingly, sperm prepared using Androcoll-B showed the highest BCF and lowest ALH, values which indicates that this treatment yielded fewer hyperactive sperm.

The second objective was to evaluate the effects of metabolic regulators (MR) on embryo development in vitro of gender-selected embryos. In experiment 2 following in vitro fertilization, Androcoll-B treated-sperm yielded embryos with significantly delayed cleavage rates at 36 hours post IVF, but this difference was no longer apparent during subsequent development to blastocysts. According to Alomar et al. (2008) early cleavage rates are associated with male embryos. Since blastocyst rates of the Androcoll-B treated group became similar to other groups, we might speculate that the pool of sperm that Androcoll-B separates may be skewed for X-bearing sperm i.e. female embryos. Also, given that Androcoll-B consistently resulted in sperm having an indication of low hyperactivity we may suggest that this motility variable may provide a marker for sex-specific spermatozoa. Contrary to Jeulin et al. (1986), our results indicate that the low ALH was not necessarily correlated with poor embryo development. Although there were significant differences in terms of blastocyst rates by day 8 post IVF, the group with the lowest blastocyst yield was the Old Bovipure group. New Bovipure is a modified formula that improves in vitro fertilization outcomes (Dr. Niläng-Laessker, personal communication). Our results corroborate that the new formulation of this product yields higher embryo rates compared with the original formula.

Our results regarding the effects of metabolic regulators on gender-selected embryos were not expected. It is important to mention that we did not sex the embryos, but they were fertilized by X-sorted semen. Thus, presumptive female embryos treated

with MR showed reduced development from morula to blastocyst stages. Further, MR treated embryos had substantially reduced blastomere counts. Altogether, our results using MR, at least using the current dose, indicate that they do not improve embryo development. Although our previous findings (Chapter Four) indicate that MR yielded better embryo development rates, using MR for presumptive female embryos needs to be tested further. According to Garcia-Herreros et al. (2012) female embryos show a reduced expression for glycolytic gene markers compared to male embryos. In addition, Sturmey et al. (2010) have indicated that there is a nutrient specificity based on embryo gender, particularly for amino acids. Therefore, it is possible that providing the current dose of MR, we surpassed a threshold for glycolysis-related gene alteration resulting in delayed development and overall reduced quality of MR-treated female embryos. However, a very interesting observation was that, within the pool of reduced quality embryos, usually one or two embryos presented a similar quality compared with untreated female embryos or the internal quality control. We speculate that those embryos may have been male, given that current sperm sorting methods have 90 % efficiency. Future experiments using MR at the current concentration, but using only male embryos may corroborate this hypothesis.

CONCLUSION

Androcoll-B and New Bovipure sperm fractionation methods resulted in improved computerized motility parameters that were positively correlated with embryo development. Reduced ALH was inversely related to blastocyst development in the Androcoll-B treated group. Subtle differences among sperm separation methods were detected as early as 36 hr post fertilization and may reflect different iso-pynic points for

each product and separation of different sperm pools reflected not only in motility differences, but also in rates of development. Addition of two metabolic regulators, PES and DNP in combination, reduced overall blastocyst rates of presumptive female embryos. Future studies are needed on the use of these metabolic regulators in combination with other important media components to study the metabolic requirements of embryos by gender.

ACKNOWLEDGMENTS

The authors would like to thank the Consejo Nacional de Ciencia y Tecnología (CONACyT, México) as well as the Department of Animal Sicence (Cornell University) for providing financial support to VAA during his PhD program. Thanks to Cargill Wyalusing, PA for providing ovaries. We also appreciate the kind support from Genex Cooperative Inc., Ithaca, NY for donating semen. Thanks to Dr. Cohen's lab for providing access to and advice for epifluorescence microscopy. Special thanks to Professor Jane Morrell from the Swedish University of Agricultural Sciences, Uppsala, Sweden for kindly providing Androcoll-B, and Dr. Anna Niläng-Laessker from Nidacon International AB, Mölndal, Sweden for kindly providing Bovipure products.

REFERENCES

Albuz, F. K., M. Sasseville, M. Lane, D. T. Armstrong, J. G. Thompson and R. B. Gilchrist. 2010. Simulated physiological oocyte maturation (SPOM): A novel in vitro maturation system that substantially improves embryo yield and pregnancy outcomes. Hum. Reprod. 25:2999-3011.

Alomar, M., H. Tasiaux, S. Remacle, F. George, D. Paul and I. Donnay. 2008. Kinetics of fertilization and development, and sex ratio of bovine embryos produced using the semen of different bulls. Anim. Reprod. Sci. 107:48-61.

Block, J., C. Wrenzycki, H. Niemann, D. Herrmann and P. J. Hansen. 2008. Effects of insulin-like growth factor-1 on cellular and molecular characteristics of bovine blastocysts produced in vitro. Mol. Reprod. Dev. 75:895-903.

Carrell, D. T., P. H. Kuneck, C. M. Peterson, H. H. Hatasaka, K. P. Jones and B. F. Campbell. 1998a. A randomized, prospective analysis of five sperm preparation techniques before intrauterine insemination of husband sperm. Fertil. Steril. 69:122-126.

Carrell, D. T., P. H. Kuneck, C. M. Peterson, H. H. Hatasaka, K. P. Jones and B. F. Campbell. 1998b. A randomized, prospective analysis of five sperm preparation techniques before intrauterine insemination of husband sperm. Fertil. Steril. 69:122-126.

Claassens, O. E., R. Menkveld and K. L. Harrison. 1998. Evaluation of three substitutes for percoll in sperm isolation by density gradient centrifugation. Human Reproduction. 13:3139-3143.

Cran, D. G., L. A. Johnson, N. G. Miller, D. Cochrane and C. Polge. 1993. Production of bovine calves following separation of X- and Y-chromosome bearing sperm and in vitro fertilisation. Vet. Rec. 132:40-41.

De Vos, A., Z. P. Nagy, H. Van de Velde, H. Joris, G. Bocken and A. Van Steirteghem. 1997. Percoll gradient centrifugation can be omitted in sperm preparation for intracytoplasmic sperm injection. Human Reproduction. 12:1980-1984.

Garcia-Herreros, M., I. M. Aparicio, D. Rath, T. Fair and P. and Lonergan. 2012. Differential glycolytic and glycogenogenic transduction pathways in male and female bovine embryos produced in vitro. Reprod. Fertil. Dev. 24, 344–352.

Holm, P., P. J. Booth, M. H. Schmidt, T. Greve and H. Callesen. 1999. High bovine blastocyst development in a static in vitro production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology. 52:683-700.

Jeulin, C., D. Feneux, C. Serres, P. Jouannet, F. Guillet-Rosso, J. Belaisch-Allart, R. Frydman and J. Testart. 1986. Sperm factors related to failure of human in-vitro fertilization. Journal of Reproduction and Fertility. 76:735-744.

Lu, K. H. and G. E. Seidel Jr. 2004. Effects of heparin and sperm concentration on cleavage and blastocyst development rates of bovine oocytes inseminated with flow cytometrically-sorted sperm. Theriogenology. 62:819-830.

Matas, C., P. Coy, R. Romar, M. Marco, J. Gadea and S. Ruiz. 2003. Effect of sperm preparation method on in vitro fertilization in pigs. Reproduction. 125:133-141.

Morrell, J. M. 2006. Update on semen technologies for animal breeding. Reprod. Domest. Anim. 41:63-67.

Morrell, J. M. and H. Rodriguez-Martinez. 2010. Practical applications of sperm selection techniques as a tool for improving reproductive efficiency. Vet. Med. Int. 2011:894767.

Mousset-Siméon, N., N. Rives, L. Masse, F. Chevallier and B. Mace. 2004. Comparison of six density gradient media for selection of cryopreserved donor spermatozoa. J. Androl. 25:881-884.

Parrish, J. J., J. Susko-Parrish, M. A. Winer and N. L. First. 1988. Capacitation of bovine sperm by heparin. Biol. Reprod. 38:1171-1180.

Pertoft, H. 2000. Fractionation of cells and subcellular particles with percoll. J. Biochem. Biophys. Methods. 44:1-30.

Pertoft, H., T. C. Laurent, T. Låås and L. Kågedal. 1978. Density gradients prepared from colloidal silica particles coated by polyvinylpyrrolidone (percoll). Anal. Biochem. 88:271-282.

Samardzija, M., M. Karadjole, I. Getz, Z. Makek, M. Cergolj and T. Dobranic. 2006. Effects of bovine spermatozoa preparation on embryonic development in vitro. Reprod. Biol. Endocrinol. 4:58.

Scott, L. and S. Smith. 1997. Mouse in vitro fertilization, embryo development and viability, and human sperm motility in substances used for human sperm preparation for assisted reproduction. Fertil. Steril. 67:372-381.

Soggiu, A., C. Piras, H. A. Hussein, M. De Canio, A. Gaviraghi, A. Galli, A. Urbani, L. Bonizzi and P. Roncada. 2013. Unravelling the bull fertility proteome. Mol. Biosyst. 9:1188-1195.

Sturmey, R. G., P. Bermejo-Alvarez, A. Gutierrez-Adan, D. Rizos, H. J. Leese and P. Lonergan. 2010. Amino acid metabolism of bovine blastocysts: A biomarker of sex and viability. Mol. Reprod. Dev. 77:285-296.

Thys, M., L. Vandaele, J. M. Morrell, J. Mestach, A. Van Soom, M. Hoogewijs and H. Rodriguez-Martinez. 2009. In vitro fertilizing capacity of frozen-thawed bull spermatozoa selected by single-layer (glycidoxypropyltrimethoxysilane) silane-coated silica colloidal centrifugation. Reprod. Domest. Anim. 44:390-394.

Xu, J., S. A. Chaubal and F. Du. 2009. Optimizing IVF with sexed sperm in cattle. Theriogenology. 71:39-47.

Xu, J., Z. Guo, L. Su, T. L. Nedambale, J. Zhang, J. Schenk, J. F. Moreno, A. Dinnyés, W. Ji, X. C. Tian, X. Yang and F. Du. 2006. Developmental potential of vitrified holstein cattle embryos fertilized in vitro with sex-sorted sperm. J. Dairy Sci. 89:2510-2518.

TABLES

Table 1. The effect of four colloidal-based sperm separators on sperm motion parameters using computer-assisted semen analysis (CASA).

Treatments	VAP*	VSL*	VCL	ALH	BCF	STR	LIN*
Percoll	150.3 ± 14.9	144.7 ± 15.0	211.1 ± 15.5	6.4 ± 0.3[a,b]	42.4 ± 1.6	95.0 ± 1.7	68.0 ± 3.5
Old Bovipure	145.7 ± 19.6	140.7 ± 18.5	207.4 ± 28.3	7.0 ± 0.3[a]	42.7 ± 2.3	96.3 ± 0.3	67.7 ± 0.3
New Bovipure	162.7 ± 14.0	157.5 ± 14.0	229.4 ± 16.5	7.1 ± 0.3[a]	43.9 ± 1.1	96.0 ± 0.6	67.3 ± 2.0
Androcoll-B	151.9 ± 12.0	148.3 ± 12.0	200.7 ± 13.3	5.7 ± 0.0[b]	43.5 ± 1.0	97.0 ± 0.6	73.0 ± 1.5

Data are presented as Mean ± SEM. N=3.

[a, b] Means ± SEM not sharing the same letter were significantly different (P< 0.05).

*Asterisk indicates that there was a trend among groups (P ≤ 0.1)

VAP: Smoothed Path Velocity (µm/s)

VSL: Straight Line Velocity (µm/s)

VCL: Track Velocity (µm/s)

ALH: Amplitude of Lateral Head Displacement (µm)

BCF: Beat Cross Frequency (Hz)

STR: Straightness (ratio of VSL/VAP)

LIN: Linearity (ratio of VSL/VCL)

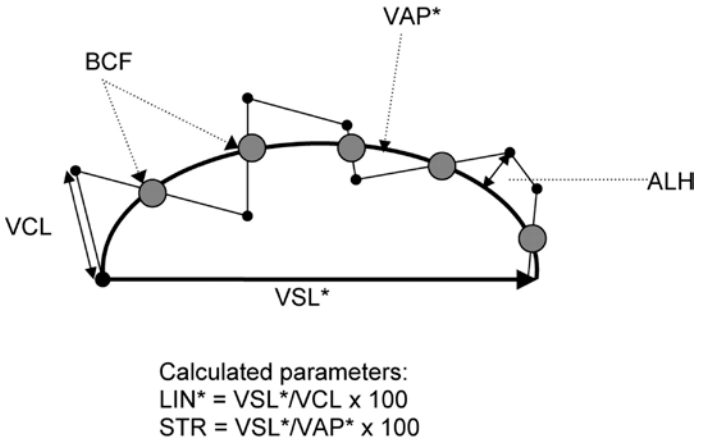

Table 2. The effect of four colloidal-based sperm separators on embryo development using traditional frozen semen for IVF.

Group	Total oocytes	Cleavage %	Morula[1]%	Blastocyst[1] %
Percoll	42.6 ± 0.9	87.7 ± 5.7[a]	88.1 ± 2.1	53.8 ± 4.1[ab]
Old Bovipure	43.4 ± 1.5	81.5 ± 5.9[ab]	86.8 ± 2.2	43.9 ± 4.8[b]
New Bovipure	42.0 ± 2.2	79.9 ± 6.8[ab]	87.6 ± 3.4	58.6 ± 2.2[a]
Androcoll-B	42.2 ± 1.1	74.9 ± 3.6[b]	82.4 ± 6.4	54.3 ± 8.3[ab]

Mean ± SEM from 5 replicates. Means within a column not sharing the same letter are significantly different ($P < 0.05$).

[1]Morula and blastocyst rates were calculated as the percent of the total embryos cleaved to account for the treatment effects.

Table 3. The effect of metabolic regulators on development of gender-selected embryos after IVF.

Group[a]	Total oocytes	Cleavage %	Morula[1]%	Blastocyst[1*]%
MR-SSS	41.3 ± 0.9	79.0 ± 2.0	85.7 ± 1.5	38.6 ± 2.9
SSS	40.7 ± 0.7	80.3 ± 0.5	90.9 ± 4.7	41.7 ± 3.1

[a]MR-SSS = Female embryos treated with metabolic regulators PES 0.3 μM and DNP 10 μM; SSS = Female embryos without metabolic regulator treatment.

[1]Morula and blastocyst rates were calculated as the percent of the total embryos cleaved to account for fertilization effect.

[*]There was a tendency for difference between groups (P = 0.06).

Mean ± SEM from 4 replicates.

FIGURES

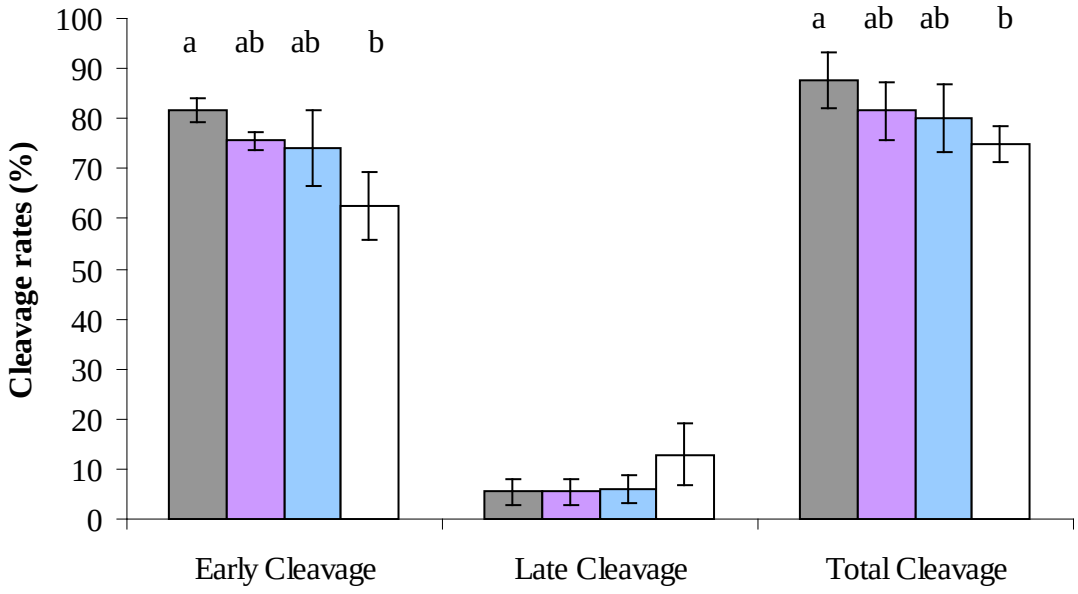

Figure 1. The effect of four colloidal-based sperm separators on in vitro embryo cleavage rates.

Gray bars= Percoll; Purple bars= Old Bovipure; Blue bars= New Bovipure and Open bars= Androcoll-B. Mean ± SEM cleavage rates not sharing the same letter are significantly different among groups ($P < 0.05$). Early cleavage was assessed 36 hours post in vitro fertilization and late cleavage along with the total cleavage rates were assessed by 48 hours post in vitro fertilization.

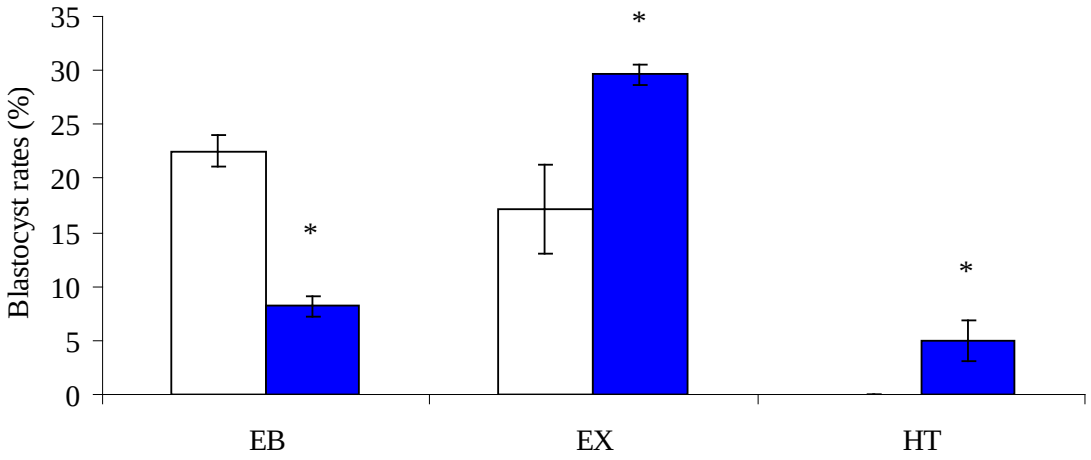

Figure 2. The effect of metabolic regulators on embryo development of embryos from sexed semen.

Blastocyst rates were assessed at day 8 post IVF. Open bars represent the gender-selected embryos treated with metabolic regulators PES 0.3 μM + DNP 10 μM starting at day 5 post IVF. Blue bars represent gender-selected embryos without any treatment. Mean ± SEM blastocyst development rates marked with an asterisk were significantly different between groups.
EB = Early blastocyst
EX = Expanded blastocyst
HT = Hatched blastocyst

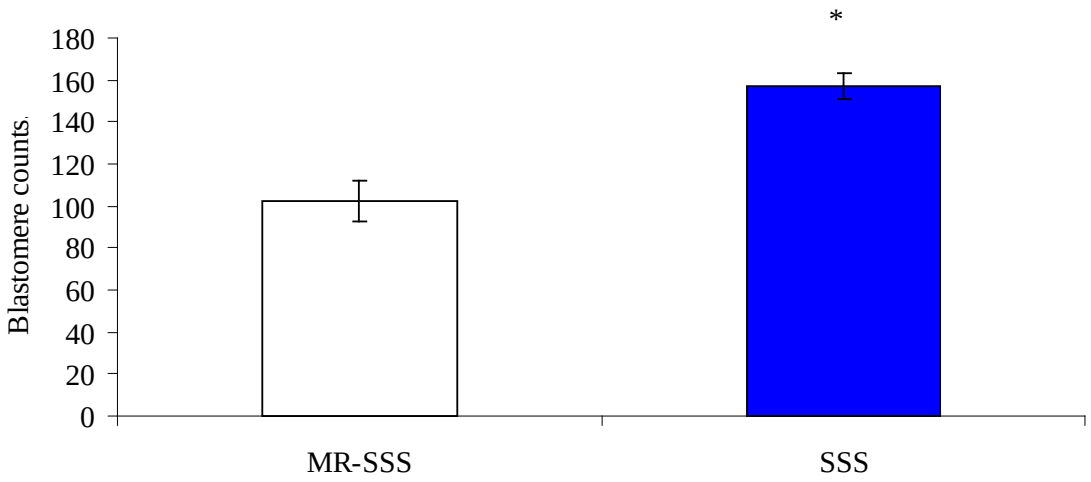

Figure 3. The effect of metabolic regulators on blastomere counts of gender-selected embryos.

Open bars represent the gender-selected embryos treated with metabolic regulators PES 0.3 μM + DNP 10 μM starting at day 5 post IVF (MR-SSS). Blue bars represent gender-selected embryos without any treatment (SSS). A total of N= 86 Expanded-stage embryos were analyzed. Mean ± SEM blastomere counts marked with an asterisk were significantly different between groups. Counts were assessed at day 8 post IVF.

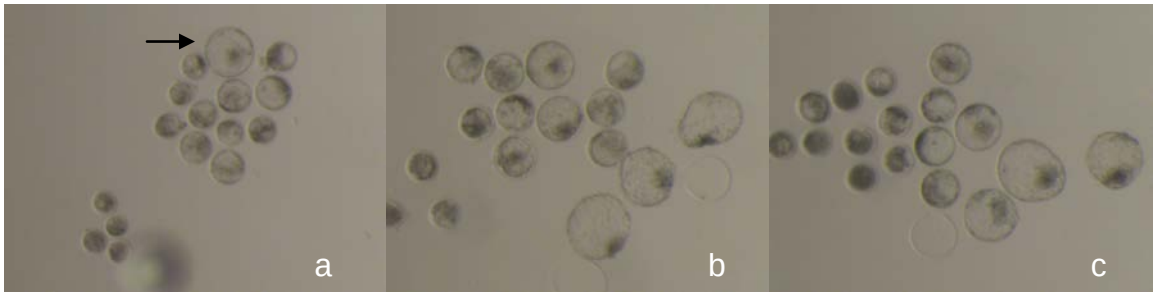

Figure 4. The effect of metabolic regulators on embryo morphology.

Panel a = gender-selected embryos treated with metabolic regulators PES 0.3 μM + DNP 10 μM starting at day 5 post IVF (MR-SSS). Arrow = Normal expanded blastocyst. Panel b represents gender-selected embryos without any treatment (SSS). Panel c represents control embryos fertilized using conventional semen.

INTEGRATED SUMMARY AND FURTHER REMARKS

Over the last three decades dairy cattle reproduction has been facing several metabolic challenges as a consequence of genetic selection for improved milk production traits. Assisted Reproduction Technologies (ART) have the potential to solve several issues the modern dairy cow is presenting to farmers. One example is the production of embryos in vitro. Embryo transfers using in vitro produced embryos (IVP) would have an advantage over conventional breeding methods since ovulation, fertilization and early embryonic stages would be bypassed, thereby enhancing the likelihood of embryo survival to implantation and hence improved pregnancy rates. However until today, based on several studies, we are not able to produce embryos of similar quality in vitro as compared to in vivo derived embryos. Better emulation of the molecular environment the embryos experience in transit to the uterus before implantation is necessary in order to produce good quality embryos in vitro. The overall objective of this dissertation was to study the effects of chemical additives that regulate metabolism at critical stages during this process and may enhance bovine embryo production in vitro.

Metabolic processes occurring at preimplantation embryo stages and important updates during oocyte in vitro maturation along with respective sperm interactions during fertilization were reviewed in Chapter Two. As a result of the literature review, important new information led to further research with our in vitro embryo production system that resulted in a remarkable 10 % increase in blastocyst rates. Due to the strong impact polyunsaturated fatty acid CLA has as a nutraceutical within the public and research community, a systematic study was conducted on the effects of conjugated linoleic acid (CLA) isomers on embryos produced in vitro (Chapter Three). The addition of CLA to morula-stage embryos that were subsequently cultured

for 36 hrs before cryopreservation resulted in embryos with higher survival and better developmental rates post-thaw when compared to other groups. Enhancing glucose uptake and metabolism in morula stage embryos by providing an assisted metabolism approach is presented in Chapter Four. The addition of phenazine ethosulfate (PES) and 2, 4-dinitrophenol (DNP) resulted not only in higher embryo development and better quality, but also the embryos better withstood the cryopreservation procedures. Chapter Five presents the results of a study on the effect of four sperm separation products using different fractionation methods. Embryos derived from sperm fractionated by New Bovipure and Androcoll-B showed the highest blastocyst rates compared with Percoll and Old Bovipure groups. Surprisingly, we also found that addition of PES and DNP to presumptive female embryos from sex-sorted semen at morula stages resulted in delayed development and poor morphology as compared with untreated presumptive female embryos. More studies are necessary to define and optimize methods of culture for gender-selected embryos in vitro. Critical information has resulted from this dissertation that will certainly contribute to a better understanding and improvements in bovine IVP and to shape future research.

The future demand for products from livestock species is predicted to increase dramatically as the current world population is predicted to rise from 7 to 9 billion people by the year 2050. For this reason it is necessary to define novel strategies for meeting those rather intimidating, demands. Assisted Reproduction Technologies have the potential to improve not only pregnancy rates, but also would provide producers opportunities to intelligently allocate types of genetics and gender specificity in order to improve their current management programs. As we may also reach the limits of the carrying capacity of our current production systems, genetically modified animals and plants are widely thought of as the potential solution. However, research on sustainable agriculture will play a fundamental role in how to integrate novel livestock

production systems with minimal environmental impact. The future of ART in light of genetically modified animals will involve a close relationship with the novel discipline area of nutrigenomics in order to exploit the full genetic potential of new genetic strains.